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# CHEMICAL EMBRYOLOGY

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**DEDICATED**  
**to the Memory of**  
**LOUIS RAPKINE**

## Translator's Preface

The English translation of Jean Brachet's *Embryologie Chimique*, presented to students and researchers in biology in 1950, forms a fitting climax to the development of this field, which began with the studies and writings of Jacques Loeb in 1900. Loeb by his books and papers on the chemical aspects of fertilization provided a great stimulus for graduate students to undertake problems in the chemistry of embryonic development.

A second surge in the direction of chemical studies was initiated by Joseph Needham's *Chemical Embryology* in 1931, and the subsequent researches into the chemical nature of the amphibian organizer. Another contribution by Needham in 1942 brought together the facts and theories of development in *Biochemistry and Morphogenesis*.

Now Brachet has taken the brilliant step of organizing the vast amount of biochemical data and the various theories of development into a comprehensive, critical, and complete story of embryonic development, beginning with the formation of the egg in the ovary and carrying it through the essential stages of differentiation. The 1945 French edition of *Embryologie Chimique* has been widely used in this country, and it is to the credit of Interscience Publishers, Inc., that they undertook the task of making this valuable book available to the large number of students who have been awaiting with eagerness an English translation.

May, 1950  
Columbia University, New York City

L. G. BARTH

## Preface to the First French Edition

Few biological phenomena are more interesting than embryonic development. In the making of the individual, we can actually see a progression from a simple condition to a more complex one, and we can study the never-ending perfecting of structures and the foundation and building of the most delicate ramifications of organs and systems. This is the only case in which life manifests itself so strongly that, in the words of Albert Brachet, it is, in itself, "creative of form and function." The relative simplicity of the infertile or just fertilized egg makes it a nearly perfect material for a study of the basic properties of living matter, and the fact that embryonic stages can be experimentally analyzed further enhances the interest that the zygote from which they evolve holds for us. Finally, an embryo is, by and large, a closed system capable of synthesizing its protoplasm from the reserves stored up in its cells, taking from the external environment only basic substances like water, oxygen, and mineral salts, making it thus more easily susceptible of chemical analysis.

The study of embryology, descriptive at first, was not long in becoming experimental. Without a doubt, experimental (or "causal") embryology is one of the scientific triumphs of this century, and certainly its future course will be a long and successful one. However, it cannot be said that this branch of embryology alone satisfies all of one's questions. The terminology of experimental embryology is often vague, precisely because of a lack of sufficient physical and chemical knowledge about embryos; above all, current ideas about germinal localization, induction, fields, and gradients ought to be founded as much as possible on a basis of precise chemical information. Chemical embryology should fill the gaps and provide an exact material basis for those "entities" to which experimental embryologists have had recourse in explaining developmental phenomena. So, we hate to admit that in spite of the important progress that it has made in the past ten years this branch of the science is not yet able to answer all the questions currently being asked of it.

The field may be said to have begun existence as a separate discipline with the appearance in 1931 of Joseph Needham's monumental work on chemical embryology, in which were collected and coordinated the facts discovered until then by those workers who had been interested in the chemical nature of embryos. In the past decade, however, events have taken quite a different turn and the orientation of research in the field has changed decidedly. In the beginning, the egg or the embryo was considered only as a source of convenient material for biochemists interested in studying certain chemical phenomena under comparatively simple conditions; now, the egg itself and its morphogenesis constitute the focal problem, which the infinitely valuable techniques of chemistry and physics are called upon to help in solving. These sciences furnish the present-day embryologist with the tools needed to attack the fundamental questions of form and function in the course of development.

Chemical embryology already has to its credit many valuable contributions, and it is certainly safe to predict that the future will produce other important discoveries in the field. It would seem that now is a good time to make up a provisional balance sheet of the results to date, research having been halted or slowed down because of the war; fresh facts will again pile up in rapid succession, as they always do when conditions are back to normal.

The difference between the guiding concepts in the field ten years ago and those which we have at present becomes clear if we compare the plan of Needham's book with the plan of this one. The English author wrote his work from the point of view of the biochemist, examining the distribution of various substances in the egg and following their quantitative variations and metabolism. We have, on the contrary, decided to use the classic form of embryology textbook, beginning with a consideration of the reproductive cells and ending with the embryo complete as to all its fundamental organ systems.

This book is intended primarily for biologists and it is hoped that it will attract some of them toward the fertile field of chemical embryology. In particular, it was planned to make this field of research understandable to any one with some biological training, but too great simplification is not possible when a considerable knowledge of the fundamental sciences must be presumed and where the facts must still be examined critically. The discussion

of experimental work was with these ideas in mind, but the concluding portions of certain sections were deliberately given over to hypothetical considerations with the hope of stimulating further research to prove or disprove their validity. The bibliography which follows the text will prove useful to any one wishing to pursue certain points. Although it is fairly extensive, it is by no means complete, because it has deliberately been restricted to papers whose purpose is to determine the physical and chemical bases of morphogenesis. Those who wish a more complete bibliography will find one, up to 1931, in Needham's *Chemical Embryology*, and, for the period of 1931 to 1941, in the very good review articles published in *Annual Review of Biochemistry*, *Annual Review of Physiology*, and *Fortschritte der Zoologie*.

It seemed useful at times not to limit the discussion strictly to those results obtained from studies of the egg as such, since the ovum is a cell, after all, and consequently presents a series of problems in cellular physiology. There is, for example, the problem of the relative roles of the nucleus and the cytoplasm in metabolism, or the problem of cell division. It seemed natural, in such cases, to look for correlative evidence among the results acquired from studying ordinary tissue cells or the protozoa.

Because the length of this book would have become excessive and because there already exist very good recent works on the subjects concerned, it was naturally necessary to leave out fundamental facts established by descriptive and experimental embryology and by biochemistry. However, basic points are reviewed in succinct fashion, wherever it was considered desirable, thus preserving the unity of the book as well as making it unnecessary for the reader to refer continually to special works.

The circumstances under which this book appears bring to mind those which introduced *L'Oeuf et les Facteurs d'Ontogénèse* of Albert Brachet, since, in both cases, a war sharply curtailed the rapid development of a new science. Without pressing the analogy further, it seems appropriate to dedicate this book to the memory of one who was, for the writer, at once a father, a friend, and a teacher.



## From the Preface to the Second French Edition

The second edition of this book follows closely the first one: we have introduced only small changes of details, attempting to take into account as extensively as possible the English scientific literature published during the war years. Unfortunately, this literature is not even now completely available to us, especially for the years 1943 to 1944. The chief work in the field of chemical embryology during the war years seems to be the new book by J. Needham, *Biochemistry and Morphogenesis*. This important publication is of the highest interest for the originality of the concepts it expresses, as well as for its wealth of documentation.

It is with great satisfaction that we realize that Needham, as we have done, places the emphasis on biochemical and biophysical investigations that have contributed to the elucidation of the problem of morphogenesis, and that he has relegated purely biochemical facts somewhat into the background. The conclusions at which Needham arrives coincide in almost all points with the view which we have developed in the present book.

J. B.

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## CHAPTER 1

### Problems—Methods

There are few of the fundamental problems in embryology which at the present time have not been subjected to a biochemical analysis. Even so, the questions for which we shall attempt to find answers are numerous and varied. In the first place we will examine in summary fashion the chemical basis for the *determination of sex* and this study will deal mainly with simple forms where it is relatively easy to grasp the chemical reactions which steer the potentially bisexual cell toward one sex or the other. The problem of secondary sexual characteristics which concerns relatively complex organisms belongs to the realm of physiology. We will only touch upon it while we will attempt at greater length to find out whether sex is determined by differences in respiratory metabolism or by other reactions.

In sexual forms the reproductive organs produce eggs and spermatozoa and the *formation of these gametes* has stimulated innumerable cytological investigations. The role of the chondrioma, of the vacuome, of the Golgi apparatus, of the nucleoli in the formation of yolk has been discussed many times but the problem has its chemical as well as cytological aspects. The egg in its growth phase is characterized by an intense synthetic metabolism and, in addition to the protein or lipoprotein yolk, fat and glycogen reserves accumulate. We have then to consider what we know of the mechanism of these syntheses and this will bring us once more to the controversial question of the participation of the nuclei and of the mitochondria. Unfortunately the study of the metabolism of the oocyte has not advanced as far as we would like and we can only propose very incomplete solutions.

Fusion of the gametes occurs at the time of *fertilization* which has been a subject of numerous chemical investigations and we can

profitably compare these results with others obtained from purely experimental studies, for example, the comparison of fertilization and parthenogenesis. The stimulation of the egg without the participation of the spermatozöon allows us to determine the role of the latter in the chemical reactions which take place in the egg.

Fertilization of the egg is immediately followed by division into cells (blastomeres) which become smaller and smaller. At this time an uninterrupted series of cell divisions begins and we are faced with the problem of integrating the physical and chemical mechanism of mitosis. We can not hope to give at this time a complete explanation but we can at least analyse the phenomenon in the light of the fundamental properties of the various substances which enter into the process during division. The latter entails a reduplication of the chromosomes and consequently a synthesis of chromatin and since mitoses succeed one another rapidly and in rhythm during segmentation of the egg, we can study the mechanism of this synthesis under optimal conditions. This naturally leads to an examination of the physiological role of the most important components of chromatin, the nucleic acids.

*Gastrulation*, which follows segmentation, is characterized by important cell migrations (morphogenetic movements) which are accompanied by growth of the developing egg. The embryo next goes through a phase of organogenesis followed by the histological and cytological differentiation of the formed organs. Are these various stages associated with particular metabolism? Is there any reason to believe that growth and morphogenetic movements require a large amount of energy? What are the chemical reactions which are at the basis of the phenomena of differentiation? Such are the questions for which we shall try to find answers, even though they are only partial ones.

We have finally to consider several points of special embryological significance. We know that experimental embryology has established that the separation of the first two blastomeres in the sea urchin gives rise to two complete embryos, though the normal fate of each of these two cells would be to form only a half embryo. In this case there occurs a "regeneration" of the part which is lacking and we call this *regulation*. In other species this regulation is less striking and the isolated blastomere is incapable of forming a complete embryo so that these eggs show a *mosaic* of organ-forming substances or of

germinal localizations. It should be added, however, that, even in the mosaic type egg, there is always a certain degree of regulation but the importance of this varies in different species. We thus have to find out whether these two types of eggs differ fundamentally from each other from the point of view of chemical architecture. Have regulative eggs a relatively homogeneous cytoplasm as contrasted to mosaic eggs which show a framework of distinct substances corresponding to differences in germinal localization? This is a point which must be thoroughly examined.

We know that one of the most powerful agents in morphogenesis in the vertebrates is the induction of organs by organizers. Thus it is that the formation of the nervous system is possible only if the cells are stimulated by the inductor coming from underlying tissue. What is the chemical nature of these organizers and what is their metabolism; what is the mechanism by which they exert their effect, and how do the neighboring cells react to their influence? We see that in this field, too, we will have to raise important questions, the solution of which will greatly clarify the mechanisms of morphogenesis.

In this overall view we have reviewed the extent of the problems involved in chemical embryology. Those who devote themselves to these problems are obliged to acquire sufficient knowledge in descriptive embryology, in experimental embryology, in biochemistry, and in biophysics. It is also necessary to master the techniques of these various disciplines. The progress attained in the last few years by chemical embryology has in fact depended to an amazing extent on refinement of methods. While chemical analysis was only practicable with several grams of substance, we could not think of attacking the problem of morphogenesis on a chemical basis. How could we have compared the respiration or the enzyme activity of tiny fragments extirpated from eggs of small size? Actually, the methods are now available and are being continually refined; thus, new problems of more and more delicate nature constantly become accessible so that henceforth we can scrutinize the innermost workings of the living cell. Thanks to these technical advances, which we owe to a large degree to the ingenuity of Linderstrøm-Lang and his collaborators (Holter, Zeuthen, etc.) as well as to Caspersson, chemical embryology has become one of the most fascinating branches of modern biology.

It is impossible by reason of their diversity to give here any more than a glimpse of the techniques used in chemical embryology,



and naturally the worker in the field should be grounded in the classical methods of descriptive embryology (examination of fresh material, technique of histological preparations) and of experimental embryology (micro-dissection, generally carried out free hand). These techniques are too well known to occupy our time at this point. Further, the methods of experimental embryology have been described in detail by Mangold and by Runnström and the latter has also summarized in excellent fashion the principal techniques of chemical embryology. We will confine ourselves to stressing some procedures which have recently been applied and shown to be particularly fruitful. Since they are in current usage it is fitting to examine both their advantages and their difficulties as well as the principal sources of errors which are involved. This treatment of the techniques will be brief and we refer the reader who wishes to familiarize himself with their application to the original sources.

Following the suggestion of Linderstrøm-Lang, we will distinguish between cytochemical and histochemical methods. The former permits the determination of the chemical constituents of a cell or a portion of a cell and gives us information concerning the chemical nature of the chromatin, of the nucleolus, of the mitochondria, etc. These techniques are characterized by their extreme sensitivity and they are the only ones which can be used when we want to analyze the composition of a cell of ordinary size. They present the difficulty of being purely qualitative, although the determination of the nucleic acids on the desired scale has been made possible by Caspersson. Furthermore it appears that the method of this investigator, which will be described farther along could be extended without difficulty to the analysis of other chemical constituents of the cell. Cytochemical techniques have been studied with very good results by Lison, who has carefully defined the causes of the errors which enter into some of them, and some useful procedures have since been published by Gersh and by Wislocki and Dempsey. We will discuss here only those methods most frequently used in chemical embryology.

The histochemical techniques have originated and have been developed especially by Linderstrøm-Lang and Holter and their collaborators at the Carlsberg Institute and by Kirk in the United States. They are much less sensitive than the cytochemical methods for they do not permit us to work on a single cell or on fragments of cells. But the much greater size of eggs makes them accessible to

these methods, which have the twofold advantage of being quantitative and very carefully designed. Because of these methods, we can study the respiration or the activity of various enzymes of the nucleus and the cytoplasm of the oocyte, of isolated blastomeres even in relatively small eggs like that of the sea urchin. The experimental errors are not appreciably greater than those of ordinary micro-methods from which they were extended. The determinations allow us to deal with variations of the order of one thousandth of a milligram ( $\gamma$ ) with a precision equivalent to that of micro-methods which give results in terms of milligrams. Most of the manipulations are simple and working in series is particularly easy. In addition, these histochemical techniques offer the advantage of following a phenomenon in time (respiration, glycolysis), while the cytochemical determinations are static and give purely topographical information. We believe that, if the methods of Linderstrøm-Lang are more difficult to master than the cytochemical procedures, and if they are longer and more complicated, they have none the less a greater future. The results which they give inspire more confidence by reason of their more solid chemical bases and it is beyond doubt that their future will make for more rapid progress in chemical embryology. This does not at all mean that cytochemical methods ought to be deliberately discarded, for they are often the only available ones, since the techniques of Linderstrøm-Lang are more particularly applicable when it is a matter of determining an enzyme whose catalytic action increases the sensitivity of the reaction to be followed. The value of cytochemical techniques is very variable and certain ones are excellent. Whenever possible, however, it is desirable to verify the results obtained by means of an appropriate quantitative histochemical method. Let us now examine some of the more widely used analytical procedures.

### 1. Cytochemical Methods

These are the only methods which allow us to determine whether the granules visible under the microscope are protein, fat, or carbohydrate in nature. As has been pointed out by Lison these techniques are valuable only if two preliminary conditions are fulfilled: (1) the chemical reaction (generally colored) used must be specific, and (2) the location of the substance in question should remain unchanged during the manipulations. As a result of this second require-

ment, the fixation of the cells should be excellent and of such a nature that the penetration of the fixing agent does not displace the substance under investigation. The *in situ* preservation of certain soluble substances such as glycogen is best when the cell is fixed according to regular cytological methods since, when agents which precipitate glycogen (alcohol for example) are used, poor preservation of the structures results. In cytochemistry we tend more and more to use Altman's method of fixation and embedding which has been modified by Gersh, Hoerr and Gersh, and Landau. It consists in dehydrating the frozen tissue at low temperatures in a very high vacuum and immersing the dried mass in melted paraffin and cutting into sections. The procedure has the advantage of reducing to a minimum the changes produced by fixing agents and of avoiding the passage through alcohols and toluol which extract the fats.

The presence of proteins can be recognized by the xanthoproteic or by the Millon reaction. One can also use successfully for the same purpose the ninhydrin reaction or that of Romieu (development of a red or violet color in the presence of concentrated orthophosphoric acid with moderate heating). Another method based on the property of *o*-diacetylbenzol forming a red-violet color with proteins (due to the  $\text{NH}_2$  groups) has been described recently by Voss. More recently, methods have been developed by Serra and by Thomas for demonstrating in microscopic sections an amino acid, arginine: the reactions are especially suited for detecting basic proteins rich in arginine and particularly the protamines and histones. These proteins are also recognizable, according to Hydén, by their strong affinity for acid dyes in acid solutions. The specificity of the dye is increased by the addition of a detergent which tends to reduce the non specific adsorption of dyes. We are indebted to Serra (1946) for an interesting investigation of the methods for the cytochemical detection of proteins, a work which may be consulted to great advantage.

The presence of lipids is detected by virtue of their staining with Sudan III, Scharlach R,\* black Sudan B, red Sudan B and blue B.Z.L. Serious difficulties are encountered when one tries to determine the nature of these lipids (glycerides, phosphatides, sterols, etc.). The question has been discussed at length in a critical fashion by Lison and we refer to his publications for full details. The most reliable results are obtained in the case of sterols (Liebermann's reaction and with

digitonin) and with phosphatides. Among the latter, certain ones give, after fixation with sublimate, an aldehyde reaction with fuchsin decolorized by sulfur dioxide. This is the "plasmal" reaction of Feulgen and Voit brought about by plasmalogen, which is a phosphatide with the peculiarity that the glycerol is combined with an aldehyde of fatty acid (acetal phosphatides of Feulgen and Bersin). We owe to Verne some interesting studies on the relations between plasmalogen and the lipids which stain with Sudan. In addition Spek has stated recently that it is possible to determine the phosphatides in the living cell after staining with vital dyes (notably with iris blue). They take up the dye, which can be intensified considerably as a fluorescent spectrum.

Investigations on *glycogen* are beset with certain difficulties because this polysaccharide is fixed *in situ* with difficulty. It can be determined by Best's method or with lugol but actually Bauer's reaction, based on the ability of polysaccharides to recolor, (after oxidation by chromic acid) fuchsin decolorized by  $\text{SO}_2$ , sulfur dioxide, is preferable. This reaction is also given by cellulose, tunicin and the glycoproteins (mucus, for example). Fortunately, the action of salivary amylase allows one to control the specificity of the reaction rather easily. The best fixatives seem to be those containing picric acid (Pasteels and Léonard, Gendre, Schabadasch).

Substances containing *sulfhydryl groups* ( $-\text{SH}$ ) are also susceptible to cytochemical detection, especially glutathione or proteins containing cysteine. Concerning the latter it is important to remember that the denaturation of proteins increases the amount of free  $-\text{SH}$  groups, and the fact that we obtain a positive reaction in a fixed cell does not necessarily mean that the sulfhydryl groups of the proteins were already present in the living state. It is possible, to a certain degree, to distinguish glutathione from proteins because of its solubility in trichloroacetic acid, and we are able to establish the presence of disulfide bonds ( $-\text{SS}-$ ) present in oxidized glutathione or cystine by treatment with cyanide which reduces these groups to  $-\text{SH}$ . These latter groups are identified by the red color which they give with ammoniacal nitroprusside. In cytochemistry it is profitable to use the reaction of Giroud and Bulliard (fixation with trichloroacetic; washing; treatment with 5% zinc acetate for 30 seconds to 1 minute; washing; treatment with 2 to 5% sodium nitroprusside) which gives a less intense color but is much more stable. We have

had satisfactory results with paraffin sections ( $30\ \mu$ ) in the case of frog ovaries when embedding was rapid.

Very recently, Chèvremont and Frédéric have described a new technique which certainly has its advantages. It is based on the fact that the  $\text{—SH}$  groups reduce ferric ferricyanide to Prussian blue. Since other substances, notably melanin, also reduce this reagent one must be sure that it really tests the  $\text{—SH}$  groups by blocking them with monoiodoacetic acid, chloropicrin, or sublimate (30–60 minutes for 1% sublimate). This method is applicable to paraffin sections after fixation by Bouin solution or by formalin. The sections are immersed for 20 minutes in three successive baths of the following mixture: 1 part of 0.1% potassium ferricyanide, 3 parts of 0.1% ferric chloride. It is important to avoid the use of any metallic instrument. The stained sections are thoroughly washed and mounted in Canada balsam. This method has not yet been tested in chemical embryology. The results of some experiments done by the writer show that it is not useful when the eggs are pigmented and that the method of fixation (trichloroacetic acid, Carnoy, Bouin, formol) modifies the reaction. Nevertheless we believe that it will be very serviceable when the optimum conditions for fixation have been determined.

We have discussed at some length the detection of  $\text{—SH}$  groups because these radicals appear to play an important role in growth and morphogenesis. There is every reason to believe that they also participate in oxidations and in carbohydrate metabolism (Hopkins and Morgan, Rapkine).

It is known that vitamin C or *ascorbic acid* is a derivative of carbohydrates which, along with the  $\text{—SH}$  groups, takes part in the phenomena of cellular reductions. These substances can also activate various enzymes which function only in the reduced state and regulate the rate of certain reactions in the cell. Ascorbic acid can be determined because of its ability to reduce acid silver nitrate to a black precipitate (Giroud and Leblond), and this method has been used on eggs, but it must be noted that its specificity is not always certain. Even though Giroud was able to control the reaction so that it was negative in animals lacking in vitamin C this test is evidently not applicable to embryonic stages. It should further be noted that ascorbic acid by this method appears in the form of a silver precipitate in the section and it is highly improbable that this substance, being

very soluble and easily extracted from tissues, would have such a precise localization within the living cell (Barnett and Fisher).

Let us go on to the cytochemical detection of *respiratory enzymes*, which is frequently used in chemical embryology. Tests are made by adding to the cells certain derivatives of the phenols which become colored upon oxidation and this is why the oxidases which are determined are often called "phenolases." Special attention has been given to the enzyme which transforms a mixture of  $\alpha$ -naphthol and dimethyl-*p*-phenylenediamine (Nadi) into indophenol blue (*indophenol oxidase*) and to *peroxidase* which oxidizes benzidine to a blue compound in the presence of hydrogen peroxide (benzidine peroxidase). Because of Keilin's work it is generally believed that indophenol oxidase can be considered identical with cytochrome oxidase or the respiratory enzyme of Warburg. Thus this enzyme is identified as one of the most important oxidative enzymes. In regard to the reaction with benzidine, it no doubt reveals all the peroxidases including true peroxidase, the cytochromes, and hemoglobin, for it is a reaction of the hemins which these substances give with variable intensity. Thus the enzymes which can be determined by cytochemistry have, to all appearances, a real physiological significance and an unquestionable interest. In spite of this favorable conclusion, we believe that the results furnished by cytochemical methods with regard to respiratory enzymes should be taken *cum grana salis*. The causes of errors with which the investigator is faced are really serious and numerous. First of all, we must take into account the fact that the colored products formed (indophenol blue, benzidine blue) are easily reduced and decolorized by the reducing systems of the cell. For example, Keilin has shown that fresh yeast gives neither of the two reactions although it is particularly rich in cytochrome oxidase and in hemins. The oxidation of the Nadi reagent and of benzidine in the presence of hydrogen peroxide can be obtained only if the reducing activity of the cells, especially that of dehydrogenases, is inactivated by heat. Thus a cell or a part of a cell does not necessarily give the phenolase reaction when it is rich in cytochrome oxidase and in hemins; it should also be kept in mind that the intensity of respiration depends on dehydrogenases as well as on cytochrome oxidase. Consequently a negative reaction does not mean that the enzyme sought for is absent and a positive reaction does not imply that the region which furnishes it has a particularly

intense respiration. There is another difficulty to which Hollande has drawn attention. It may be that the enzymes sought for are found in a diffuse state in the cell or that they are dispersed in the hyaloplasm in the form of small granules. Many of the facts, to which we will return later, show that these enzymes are bound to some very fine particles visible only with the ultramicroscope. The color formed will then be distributed in the cytoplasm in a nearly homogeneous fashion but if the cell contains granules which adsorb this color they will soon appear tinted blue even though they do not contain any phenolases. Thus we have a secondary coloration of the particles comparable to the vital staining of vacuoles by neutral red. Hollande has observed that if one stains cells with indophenol blue or benzidine blue one obtains pictures which resemble those brought about by the reactions of the oxidases. We must also take into account that the benzidine is a basic dye which will thus stain the basophilic structures in the cell and that indophenol blue gets into the lipids very quickly, giving them a violet color which is somewhat different from the usual blue color and it is necessary to distinguish this slight difference carefully. We see that many dangers lie in the path of the investigator who uses these methods. Even the ease with which they are carried out makes them dangerous, and it is not surprising that the results which they give often completely contradict those of classical chemical methods.

During the last few years, Gomori has developed an interesting method for demonstrating acid and alkaline phosphatases cytochemically and its use is rapidly spreading. Fortunately these phosphatases resist fixation by alcohol or by acetone, as well as embedding in paraffin. Sections are incubated in the presence of a phosphate ester (glycerophosphate, nucleic acid, hexose phosphate, adenosine triphosphate, etc.) and with a calcium salt; calcium phosphate precipitates *in situ* and is detected by treatment with a cobalt salt followed by ammonium sulfide. One then observes a black precipitate of cobalt sulfide in the regions where the phosphatase was present. The reader will find an excellent critical study of this method in a recent paper by Danielli and a good summary of the results obtained in the reviews of Moog (1946) and of Gomori (1946).

Let us finish with the cytochemical determination of the *nucleic acids*. Later on we shall deal again at some length with the chemical

composition, the localization, and the function of these substances. For the moment we will recall that two principal types are recognized: *thymonucleic acid* and *ribonucleic acid* with *zymonucleic acid* as its prototype. They differ chiefly in the structure of the sugar radical. Thymonucleic acid is easily demonstrated by the elegant reaction of Feulgen and Rossenbeck currently used in cytology, based on the fact that hydrolysis of thymonucleic acid results in groups capable of reacting with decolorized fuchsin. The chemical mechanism of the Feulgen reaction has recently been studied by Stacey and his collaborators: it seems clear that it concerns the transformation of desoxyribose into  $\omega$ -hydroxylevulinic aldehyde. Carefully used, the Feulgen method permits a specific and exact determination of thymonucleic acid (Brachet, Dobson, Stowell). And in case of any doubt one can run a control omitting the hydrolysis, with the result that the reaction is then negative. Brachet (as well as Serra, Stowell, and Sanders) has shown, in addition, that the reaction is negative after the sections are treated for a few minutes with an enzyme which hydrolyzes thymonucleic acid (desoxyribonuclease of Fischer and his collaborators). This finding renders very improbable the opinion of Stedman and Stedman, who believe that the Feulgen reaction is caused by an acid protein present in the chromosomes (chromosomin) and not by thymonucleic acid. The reality of this protein does not appear to be well demonstrated as yet and the ideas of Stedman and Stedman have already been subjected to several justifiable criticisms (Serra, Caspersson, Callan, Barber and Callan).

With regard to the objections which Danielli has recently made to the Feulgen reaction, they appear to be too severe: indeed all the methods at our disposal, based on very different principles, agree in showing that thymonucleic acid is found only in chromatin; furthermore the new method by Turchini, based on the reactions of sugars with fluorones, gives results that are completely comparable with those furnished by Feulgen's reaction.

Brachet demonstrated some time ago (1940, 1941) that it is also easy to determine ribonucleic acid in histological sections. The sections are treated with *ribonuclease* which specifically hydrolyzes the acid, so that the structures which contained ribonucleic acid cease to stain with a basic dye, such as toluidine blue. A comparison between a treated and untreated preparation permits one to determine immediately the structures which contain ribonucleic acid. In place



of toluidine blue, a mixture of methyl green and pyronine (Unna) can be substituted to advantage. The former selectively stains thymonucleic acid while the second is taken up by ribonucleic acid (and other basophilic substances such as the glycoproteins which however are not affected by the ribonuclease). This enzyme is easily prepared from the pancreas and can be purified because of its unusual property of resistance to boiling. The optimal conditions for the use of ribonuclease have been determined by Stowell and by Sanders, who studied the effects of various fixatives, of temperature, of pH and the nature of the buffer on the results. It is well to take note, however, that various American writers (Schultz, Mazia, Gersh and Bodian, Cohen) have observed some hydrolytic action of the crystallized ribonuclease on some fibrous proteins. This secondary effect, perhaps due to an impurity, is much slower than the intense action on ribonucleic acid. This cause of error appears to have less significance if we remember that basophilia is not affected when sections are submitted to the action of trypsin free of ribonuclease (personal communication of H. Holter); note in addition that, according to Barker and Sanders, ribonuclease after heating in mildly alkaline solutions is free of all proteolytic activity, while Brachet and Shaver have noted that the intensity of the color reaction for arginine and tyrosine is not visibly altered when a histological preparation is treated with ribonuclease.

We owe to Caspersson a recent and very delicate method for demonstrating and measuring, on an ultramicro-scale, the nucleic acids of cells. This method does not, however, discriminate between the two types of nucleic acid, but it is possible to improve it and combine it with the use of specific nucleases (desoxyribo- and ribonuclease). Caspersson has made use of the fact that the nitrogenous constituents of the nucleic acids show a strong absorption band in the ultraviolet with a maximum at about 2650 Å. This method, unfortunately expensive and delicate, allows for the measurement of the *absorption spectra* of particles with a diameter of the order of 0.1  $\mu$ . It has given important results (which we shall discuss at length) in spite of the difficulties which arise from diffraction and refraction, which sources of error, however, could be greatly reduced by taking measurements before and after the action of a specific nuclease.

Figure 1 will aid in understanding Caspersson's method. De-

pending upon circumstances, the source of light is a powerful mercury vapor lamp, *A*, cadmium arc, *P*, for short ultraviolet or a tungsten filament lamp, *B*. The light passes through a monochromator, *C*, which permits the isolation of the desired wave length and is reflected through prism *F*, in the quartz microscope *H*, *K*, *L*. At *L*, an iris diaphragm reduces the dimensions of the field considerably. The light that has traveled through the object at *I* is sent to a photo-electric cell, *R*, by means of a quartz prism, *M*. The telescope, *O*, serves to check the center of the optical system. The measuring equipment consists of a potentiometer, *U*, with an electrometer, *S*, for balancing the circuit. One can place in the path of the light a

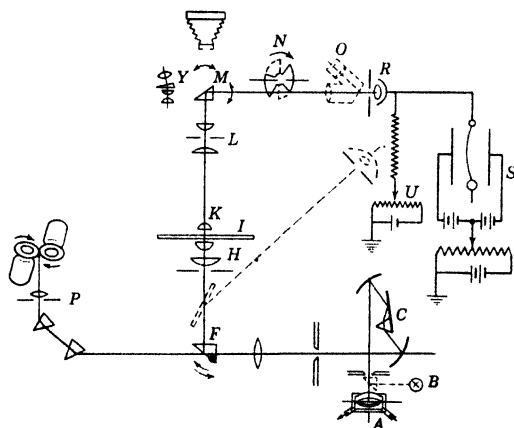


Fig. 1. Diagram of Caspersson apparatus; explanations in text (Caspersson).

sector, *N*, rotating at constant rate with an opening which can be regulated with great precision. During the measurements the object is placed in such a way that its image falls in the center of the photo-electric cell, *R*, and the electrometer is set at zero. The object is then shifted by means of a special carriage designed to bring it back in exactly the same position, and a blank measurement is made by regulating the width of sector *N* in such a way as to make the electrometer register zero again. The reading of the width of the sector gives a measure of absorption. The same manipulations are carried out for a series of wave lengths and thus the absorption spectrum is obtained.

This instrument has without any doubt a great future in micro-

chemistry, for it can be used to follow quantitatively any colorimetric cytochemical reaction, as well as to determine the amount of hemoglobin and cytochrome in single living cells. Norberg has used it as a microcolorimeter to measure very small quantities of phosphorus. Combined with the embedding technique of Altman-Gersh, it forms an ideal tool for the cytochemical study of the cell. Instruments based on the same principle but more or less simplified in construction, have been described by Gersh, Mitchell, Lavin, Stowell, *et al.*: as we see, the use of this elaborate and ingenious technique is spreading rapidly.

Finally it is important to note that Engström, a collaborator of Caspersson, recently developed an apparatus using, not only ultra-violet but also monochromatic X-radiation; this instrument, which will assuredly be of great service to cytochemistry, permits the ultramicro-determination *in situ* of various elements, providing their atomic mass is greater than that of helium. Engström succeeded in determining in histological sections from  $10^{-10}$  to  $10^{-11}$  gram of Ca or of P within 10%.

Finally let us touch on the methods which have been used to measure the pH (acidity) and the  $rH_2$  (oxidation-reduction potential) of the cell. The simplest technique consists in immersing the cell in vital dyes which change color as a function of pH (neutral red) or which are capable of reduction by the cell (methylene blue, Janus green). However the fact that these dyes stain only certain structures in the cell may result in errors; furthermore Lison has shown a "metachromatic change" which can not be distinguished from the "ionic color change" and which is due to the presence of very small amounts of esters of sulfuric acid. Thus, one can not be sure whether the color change is to be attributed to a change in acidity or to the presence of these substances. As regards the estimation of reducing power, Fischer and Hartwig (1936) have devised a technique that appears satisfactory enough, at least for the amphibian egg. Other investigators (J. and D. Needham, Rapkine and Wurmser, Chambers and collaborators) have injected indicators of pH and  $rH_2$  by means of micropipettes. The color changes are clearer, since all of the cell becomes tinted, and the range of possible dyes is very much extended, but these indicators are generally toxic for the cell and the introduction of a pipette produces an injury which may affect the pH or  $rH_2$ . In effect, cytolysis produces acidification and an increase

in reducing power of the cell. This method, however, when properly used appears to be decidedly better than the one above. Finally a few investigators (Buytendyk and Woerdeman, and especially Dorfman) have measured the pH of amphibian eggs by inserting microelectrodes (antimony, hydrogen). This method is very delicate and the results obtained are far from being in agreement. The procedure is open to criticism because the electrode causes a local cytolysis and one only measures the pH of the injured region. In the microinjection method the dye rapidly diffuses into the whole cell and this reduces the error. Furthermore there is formed around the microelectrode a precipitation membrane that gives rise to a membrane potential which may obscure the results. We see that the measurement of the pH and  $rH_2$  of the cell at the present time is both laborious and uncertain.

## 2. Histochemical Methods

These consist of the usual techniques (titration, colorimetry, manometric methods) but considerably refined. *Microtitration* was first studied by Linderstrøm-Lang and Holter and the principle corrected in carrying out the titrations with relatively concentrated solutions ( $N/30$ ) by resorting to extremely sensitive burettes and pipettes. Linderstrøm-Lang and Holter's microburette contains 100 mm<sup>3</sup> and is graduated to 0.2 mm<sup>3</sup>. It is filled with mercury, which is forced into the capillary by means of a piston controlled by a screw. The tip of the pipette is introduced into the solution that is to fill the burette and the solution is drawn up by lowering the level of the mercury by means of the piston. The latter is used to add small but definite amounts of the solution to a small container (capacity of 0.2 cc), where the titration is performed. Another type of burette is used with solutions which cannot be exposed to mercury (as, for example, in iodometric titrations). Heatley has described a microburette with a similar sensitivity, which one can make one's self. Linderstrøm-Lang and Holter have devised several types of pipettes; the most convenient is the constriction pipette, which delivers volumes of solutions from 700 to 1 mm<sup>3</sup> (Fig. 2) with a precision of 0.3 to 2%. This micropipette is made by rotating a glass tube above a microburner so that a constriction forms. The liquid is drawn up above this constriction and is then allowed to run out until it stops automatically at the level of the constriction. The contents are then

blown out, and the pipette may be calibrated either by weighing it with mercury or by titration with a microburette.

In general, a drop of 7 to 10 mm<sup>3</sup> of the substance to be titrated is introduced by pipette into the 0.2 cc paraffined container. A colored indicator is added to the required concentration, and titration is carried out with the microburette until the color is equal to that of a control tube. Stirring is very ingeniously brought about by introducing into the container a small glass ball filled with iron filings and the ball (flea) is moved up and down by an electromagnet (Fig. 3).



Fig. 2. Micropipette with Levy constriction (Linderstrøm-Lang and Holter).

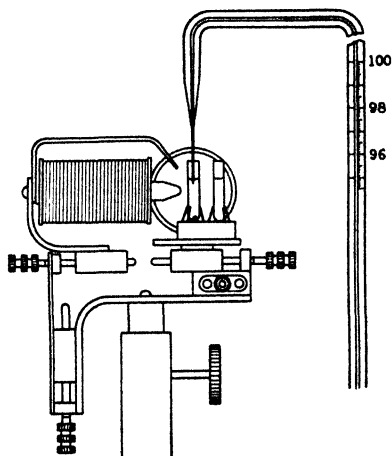


Fig. 3. Microtitration according to Linderstrøm-Lang and Holter: *right*, microburette; *center*, microtitration tubes mounted on a movable holder; *left*, arrangement for electromagnetic stirring.

Linderstrøm-Lang and Holter and their collaborators have applied their method to the determination of various peptidases, cathepsin, trypsin, ammonia, amylase, and catalase. To give an example of the sensitivity of these techniques it will only be mentioned that, if one titrates 3  $\gamma$  of ammonia (1  $\gamma$  = 1/1000 of 1 mg.), the precision is better than 0.01  $\gamma$ . Practically all of the current acidimetric and iodometric titrations in biochemistry are possible by these techniques which are 50 times more sensitive than the usual micromethods. The operations are hardly more delicate, and the loss in time negli-

ble, especially when serial determinations are to be made. Brachet has succeeded in carrying out determinations of peptidase activity on one or two isolated nuclei (germinal vesicles taken out of the oocytes of the frog).

The use of *dilatometric* methods is a further step (Linderstrøm-Lang and Lanz). They are based on the fact that in systems in which enzymic hydrolysis is occurring there are considerable changes in volume (Sreenivasaya and Bhagvat, Rona and Fischgold). A

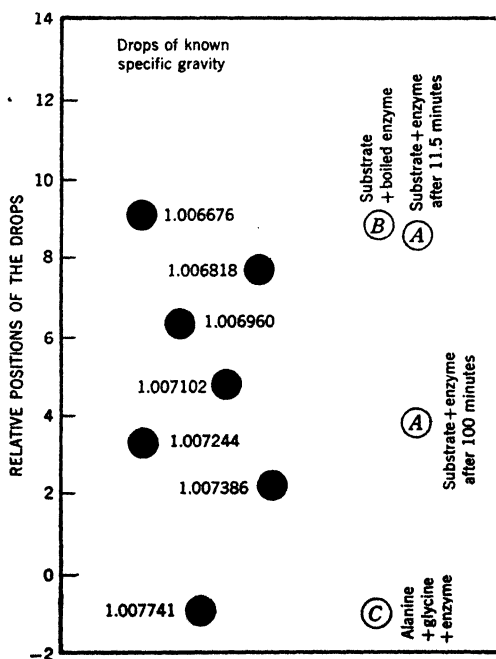


Fig. 4. Position of drops in a tube for microdilatometry (Linderstrøm-Lang and Lanz).

vertical tube filled with a mixture of kerosene and bromobenzene is placed in a thermostat which is very closely regulated to  $\pm 0.003^\circ \text{C}$ . A specific gravity gradient is established which can be standardized by the introduction into the tube of small drops ( $0.1 \text{ mm}^3$ ) of substances having known specific gravities. They come to rest at definite levels in the tube (Fig. 4, black dots). A droplet of the same volume containing a mixture of the enzyme and its substrate (for example, dipeptidase and alanylglycine) is then added, and during the

course of the enzymic reaction a continuous falling of the drop takes place; only when the reaction reaches equilibrium does the drop come to rest. This fall is measured by means of a horizontal microscope provided with an ocular micrometer. A drop composed of the enzyme inactivated by boiling and the substrate gives the zero point. Figure 4 shows the position of these drops during the course of an experiment. The advantage of this technique consists in following the enzymic reaction continuously, but it is applicable only to zero-order reaction. Its sensitivity is such that Linderstrøm-Lang and Lanz have been able to determine the dipeptidase activity of fragments of a single sea-urchin egg (diameter of about 0.1 mm). It is 50 times better than that of microtitration.

In certain cases *colorimetric* methods are equally applicable but they are limited by the size of microcuvettes. Brachet has been able to determine phosphatase activity in a few isolated nuclei of frog egg. Here one is dealing with an enzyme which catalytically liberates relatively large amounts of phosphoric acid. The micro-determination of the various phosphate compounds in a tissue generally requires a few milligrams of substance but Norberg, in making use of Caspersson's apparatus as a colorimeter, has been able greatly to increase the sensitivity of the techniques. Thanks to some very delicate methods of extraction and incineration he can now determine the nucleoprotein phosphorus in a single sea-urchin egg. This method appears very laborious, however, and its application will be doubtless restricted to cases in which it is absolutely necessary to work with extremely small amounts of living material. But at a less sensitive level the recent introduction of simplified spectrophotometers into current laboratory usage has permitted the application of sensitive and specific techniques for the ultramicro-determination of numerous compounds (phosphate esters—Lowry; purine derivatives—Kalckar *et al.*).

Finally, *manometric* methods have undergone continuous refinement in their sensitivity and convenience. It is well known that the Warburg manometric technique is currently used to measure oxygen consumption, respiratory quotient ( $\frac{\text{CO}_2}{\text{O}_2}$ ), and lactic acid production (the latter reacts with bicarbonate and the liberated  $\text{CO}_2$  is determined). Without going into the details of this already classical method we will simply point out that its sensitivity is of the order of

1 mm<sup>3</sup> of oxygen per millimeter reading on the manometric scale. For the frog's egg it is necessary to have 100 eggs in each manometer, and so it follows that this method is not applicable to a study of the respiratory metabolism of fragments of such eggs. Fischer and Hartwig (1938) have succeeded in overcoming this difficulty by decreasing the volume of the flasks which gives ten-fold increase in sensitivity. Other authors (Fenn, Duryee) are inclined to the use of volumetric measurements. Two flasks, either of the same or of unequal size, are connected by means of a fine capillary (0.3 to 0.5 mm diameter) containing a drop of colored kerosene, and this index drop is displaced when absorption of gas occurs in one of the two containers. The advantage of this method lies in the use of flasks of large dimensions without affecting the sensitivity, which depends chiefly on the diameter of the capillary. This diameter cannot be less than 0.3 mm without difficulty in manipulation. But there is a tremendous gain in sensitivity if the displacement of the index drop is followed by means of a microscope (Gerard and Hartline, Schmitt). In Figure 5 a somewhat modified Gerard and Hartline apparatus is

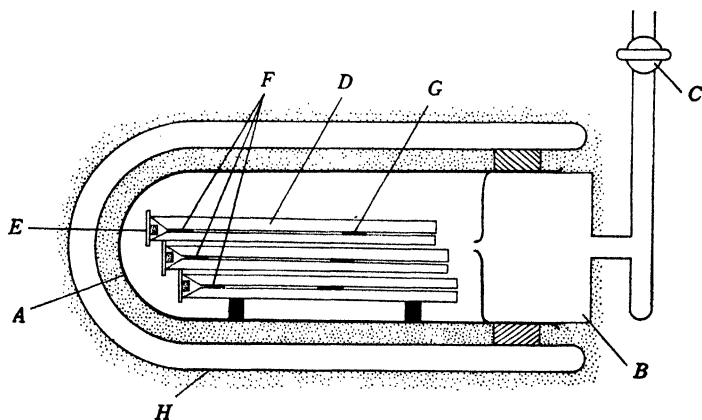


Fig. 5. Microrespirometer of Gerard and Hartline somewhat modified; explanations in text.

illustrated which has been used in chemical embryology (Waddington, J. Needham and J. Brachet, J. Brachet (1939)). A glass tube, A, is closed by a ground glass stopper, B, which communicates with the exterior through a tube provided with a stopcock, C. The tube holds three capillaries, D, constructed with a cavity which contains the



object to be studied and which is closed by a ground glass plate, E, sealed with grease. Into these capillaries is placed a thread saturated with NaOH (to absorb the  $\text{CO}_2$  produced in the case of studies on the oxygen consumption) and a droplet of kerosene, G. The diameter of the capillary is about 0.5 mm. Tube A is placed in a nonsilvered Dewar flask by means of a stopper and a constant temperature is thus attained. The entire assembly is immersed in a well-regulated thermostat. Once thermal equilibrium is established (30 to 60 minutes), C is closed and the movement of the drops, G, is followed by means of a microscope provided with an ocular micrometer. This arrangement is extremely sensitive and requires delicate handling. The capillaries must be scrupulously clean and perfectly uniform, in addition, it must be realized that the gain achieved by the use of a microscope for readings is only significant if the temperature control is very accurate. Brachet has used, by way of a test, an arrangement such that one unit of the ocular micrometer was equivalent to  $0.0002 \text{ mm}^3$ . A single axolotl egg consumed oxygen corresponding to about 50 units per minute, but it was easy to show that some significant differences resulted from simple variations in temperature of the thermostat during its heating cycle. Nevertheless, these temperature variations did not exceed  $0.01^\circ \text{C}$ , the limit for this particular experiment. Besides, it was observed that the index drop in the capillaries not containing eggs showed a displacement simulating an oxygen consumption of 10 units per minute. As a result of this high "blank" ( $0.12 \text{ mm}^3$  per hour) and of considerable thermal variation it does not appear that there is any point to measuring values less than  $0.02 \text{ mm}^3$  per micrometer unit.

An instrument suggested by the well known differential manometer of Barcroft has been used by Stefanelli. It consists of two small chambers of several cubic millimeters united by an extremely fine capillary (0.05 to 0.07 mm in diameter and even less), and an arrangement for calibration which permits correction of variations in the bore of the capillary. The apparatus is very sensitive with 1 mm of the scale corresponding to  $0.006$  to  $0.012 \text{ mm}^3$  of oxygen. There is some danger in the operation of this instrument because of the small size of the capillary. The very small dimensions of the latter suggest that the surface tension is very important.

Other apparatus, of original design, has recently been devised by Heatley, Berenblum, and Chain, who have described a microrespirom-

eter consisting of a single chamber covered with a thin sheet of mica which supports two mirrors of several square millimeter surface. This chamber is united to a manometer by means of which the mirrors on the mica are kept parallel using an optical system as an indicator. If a change in volume occurs in the chamber through an absorption or release of a gas, the mica is distorted and the mirrors form an angle. A compensating pressure is exerted by means of the manometer to return the mirrors to a parallel position and the difference between the two readings of the manometer can be used to calculate the amount of gas consumed or produced; in short the instrument functions as a Warburg manometer made very sensitive by the reduction in the volume of the chamber: 1 mm of the manometric scale corresponds to 0.008 to 0.004 mm<sup>3</sup> of gas.

We will emphasize the advantages of the operation of the Carresian diver of Linderstrøm-Lang and Holter because this instrument combines a great sensitivity with easy manipulation and low cost. It has been used successfully in chemical embryology many times and it certainly is an apparatus of great promise. Among its advantages may be pointed out the facts that it is well adapted for serial measurements and that it has been exceptionally well studied practically and theoretically. Anyone reading the papers of Holter and Linderstrøm-Lang (1943) and of Boell, J. Needham, and Rogers can construct the instrument and become familiar with its operation. By referring to Figure 6 the principle is seen to be as follows: the diver consists of a glass sphere drawn out into a neck above and into a tail below. A known volume of the substance to be studied (in general 2–3 mm<sup>3</sup>) is introduced into the diver (10–40 mm<sup>3</sup> volume) with a micropipette of appropriate size and the neck is sealed with a drop of paraffin oil of known volume and density. The diver is equilibrated in such a way as to attain a density slightly greater than the flotation liquid which fills a glass tube marked for a zero reading. This tube is connected with a water manometer adjusted by a system of syringes giving both macro- and micro-adjustments. By decreasing the pressure with the manometer the diver is raised to the zero mark, where it remains motionless, and the first reading is taken at this time. If the amount of gas present in the diver varies, the negative pressure necessary for equilibration will change. It will decrease if there is an absorption of gas and will increase if gas is produced in the diver. The pressure will depend, among other factors, on the gaseous volume in

the diver, and the smaller the latter, the more sensitive. For a diver of  $10 \text{ mm}^3$ , 1 mm on the manometric scale corresponds to  $0.001 \text{ mm}^3$  of gas, and measurements can be reproduced to nearly  $\pm 0.5 \text{ mm}$ . The reader is referred to the paper of Holter and Linderstrøm-Lang (1943) for the details pertaining to the construction, the calibration, and the

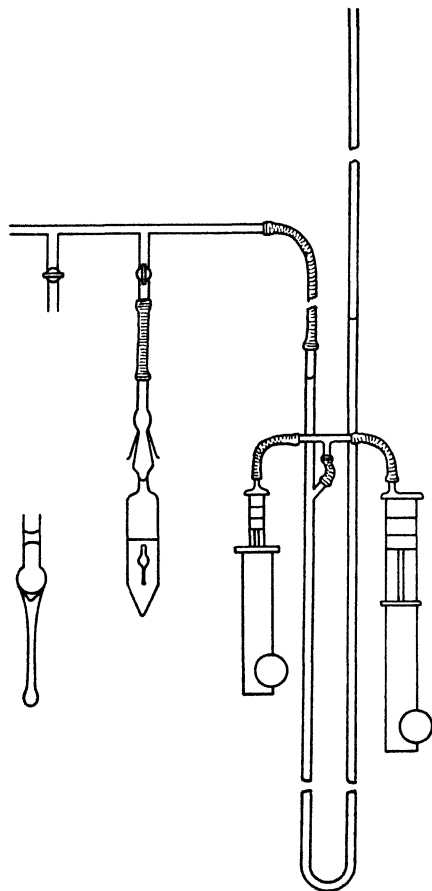
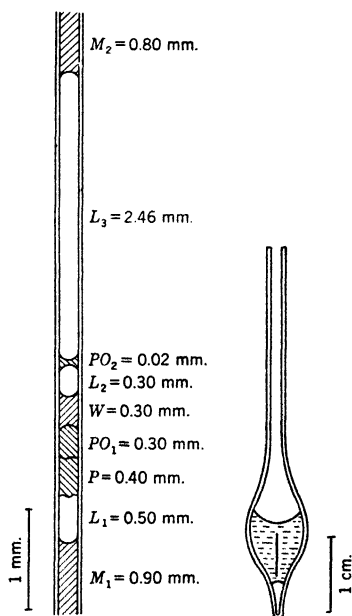


Fig. 6. Linderstrøm-Lang diver: *left*, diver with the neck sealed with a drop of paraffin oil; *right*, the diver in its container and connected with the manometer which is adjusted to different pressures by means of the syringes.

filling of the divers. If extreme sensitivity is not desired, divers of about  $50 \text{ mm}^3$  ( $1 \text{ mm} = 0.005 \text{ mm}^3$ ) can be used which can be manipulated as easily as the Warburg manometers and are 200 times more sensitive. The apparatus has been adapted by Boell, Needham, and their collaborators to the measurement of oxygen consumption, of the respiratory quotient, and of glycolysis. It is furthermore possible to

fill the diver with a definite mixture of gases or to add a reacting substance during the course of an experiment (it is placed in the neck of the diver and added when desired by submitting the glass vessel to a high pressure). With some precautions one can work under conditions of complete anaerobiosis. Brachet has used this apparatus (1944) for the microdetermination of urea by measuring the  $\text{CO}_2$  produced in the presence of urease and was able to analyze 0.5 to 1  $\gamma$  of this substance within an error of 2 to 3%. The smallest amount of urea which can be determined by this method is 0.005  $\gamma$ . After lengthy personal experience, Brachet has found the diver to be very

Fig. 7. Zeuthen microdiver (left) and the container in which it is placed (right); this container is connected to a manometer. Explanation in text; the figures indicate the length of the various drops.



much more convenient to operate and more reliable than the micro-respirometers of the Gerard and Hartline type, especially when a series of determinations is to be carried out. Anfinsen has also had recourse to the diver for the ultramicrodetermination of coenzyme, while Westenbrink has used it for cocarboxylase.

The diver just described serves admirably for measurement of oxygen consumption, of the order of 0.1 to 0.01  $\text{mm}^3$  per hour, but it is not sufficiently sensitive for lower rates of respiration and thus measurements on the eggs of marine invertebrates are not possible.

On the other hand, it has been used to good advantage in researches on fragments of the amphibian egg. Very recently, E. Zeuthen constructed a "micro diver" designed to measure amounts of oxygen less than  $0.01 \text{ mm}^3$  per hour, with an optimum of the order of  $0.001 \text{ mm}^3$  of oxygen and with the smallest quantity of gas measurable about  $0.00002 \text{ mm}^3$ . This result is attained by a drastic reduction in the size of the diver, and its manipulation naturally becomes more complicated, but the author states that it is possible to fill the divers in 20–30 minutes and to use 2–5 instruments simultaneously. Zeuthen has measured the respiration of a single egg of a polychaete worm (*Ophryotrocha puerilis*) or of a single ameba. His *microdiver* (Fig. 7) consists of a capillary, A, of uniform diameter (0.2 mm) and about 5–6 mm in length; it is immersed in a glass vessel of the type indicated in Figure 7, B, containing a flotation liquid made alkaline with NaOH in order to absorb the  $\text{CO}_2$  produced by the egg. The bottom of the diver is a layer of solid paraffin, P, covered with a drop of liquid paraffin,  $\text{PO}_1$ ; above it is placed the object to be studied in water, W, and then a small bubble of air,  $\text{L}_2$ . This is covered with a fine layer of paraffin oil,  $\text{PO}_2$ , through which  $\text{CO}_2$  rapidly diffuses.  $\text{CO}_2$  is absorbed by the flotation medium ( $\text{M}_1$ ,  $\text{M}_2$ ) which fills the two ends of the diver except for the air spaces,  $\text{L}_1$  and  $\text{L}_3$ . The diver, because of its small size, floats under water in the surrounding liquid and the pressure, exerted by means of a manometer, necessary to raise it and then just lower it slowly, is determined. The only defect of this system appears to reside in the slowness of the diffusion of  $\text{CO}_2$  through the film of paraffin and the length of the capillary to the flotation medium. It is important only in studies of quick variations in oxygen consumption and should have very little influence on determinations of long duration.

Many other types of respirometers have been described recently (Cunningham and Kirk, Tyler and Berg, Thimann and Commoner, Barth and Kirk, Scholander, Tobias, *et al.*): most of these instruments are constructed on the principle of the microrespirometer of Gerard and Hartline, but various refinements have been introduced to reduce the sources of error and to facilitate operation. We refer the reader who desires more details on this subject to the well documented review by Tobias.

Sooner or later the cytochemist meets with difficulties in the determination of the amount of living substance used for analysis.

For eggs he is often content to measure the volume. When rather large quantities are available he can determine the amount of nitrogen. There are now in existence "micro-Kjeldahls", allowing the determination with sufficient accuracy of from 1 to 10  $\gamma$  of nitrogen (Bentley and Kirk, J. Needham and Boell, Borsook and Dubnoff, Boell, Kirk and Tompkins, *et al.*). Bottelier, Holter, and Linderstrøm-Lang have devised a technique for amounts of less than 0.7  $\gamma$  of nitrogen. But, whatever the method used, a good deal of training is always necessary to get acceptable results, agreeing within 10%. When one is dealing with extremely small amounts of substance he can use Linderstrøm-Lang's stratagem. This consists in measuring the "reduced weight," that is, the weight of the tissue minus the weight of the same amount of water. The fragment of tissue is placed on a glass rod and 1 mm<sup>3</sup> of distilled water added; this drop, with the tissue, is introduced into a gradient tube similar to those used in dilatometry, and the specific gravity of the drop and tissue is determined. Thus one can "weigh" about  $5 \times 10^{-7}$  mg. It is noteworthy that the reduced weight is independent of the water content of the tissue. Although lacking this sensitivity, the quartz fiber microbalances described by Lowry and Kirk and by Craig, Gullberg, and Boyer are capable of giving good service to histochemistry: their sensitivity, reaching 0.02  $\gamma$ , allows one to weigh sections cut with a microtome.

We believe it has been useful to dwell somewhat on the principle and value of the techniques most frequently used in chemical embryology. Besides this will avoid later references to methods, which are also covered in the recent book of Glick. It is well to emphasize the vast importance of these methods since each technical advance has had a profound repercussion on our knowledge and has opened up a new field for exploration. If we cannot attack at present the quantitative study of the cell, we already have the means to attempt it on the egg. The embryologist interested in the chemical phenomena of development cannot help but feel a keen gratitude towards Linderstrøm-Lang and Holter and to Kirk who, because of their perspicacity, ingenuity, and patience, have given him some valuable tools which were formerly lacking.



## CHAPTER II

# Chemical Basis of Sex Determination

We shall concern ourselves chiefly with multicellular animals (Metazoa) in which sexual reproduction is by far most important; the chemical study of asexual reproduction remains to be done and we will have only a few words to say about it at the end of this book. Our treatment will necessarily be short, for the question of sex determination is a minor one in the realm of classical embryology. We thus refer the reader who wishes more complete information to the very complete book of Caullery (1941), to the well documented article by Hämmerling (1942), and to the important work of Hartmann (1943).

We know that the individuals of different sexes are distinguished by their reproductive glands (gonads), which give rise to the male or female reproductive cells (gametes). The fusion of the gametes, egg and spermatozoon, during fertilization stimulates embryonic development; during its course, at a specific time, the gonads are formed and the sex of the embryo becomes recognizable.

Without discussing here the biological factors that determine the sex of an individual, we will simply recall several well known facts: in a number of forms, the chromosomes play the chief role in this phenomenon; but it would be inaccurate to believe that only the heterochromosomes participate in the determination of sex, for the elegant research of Bridges has clearly demonstrated that, in *Drosophila*, the really important element is the balance between the heterochromosomes and the ordinary chromosomes (autosomes). Now, it has been shown that, in many cases factors other than the chromosomes may determine sex—those that result then from external influences. One thus can speak (Hartmann) of “genotypic” determination when sex depends on chromosomes and of “phenotypic”



determination when it is brought about by the external environment. It is this second type which functions, for example, in the polychaete *Ophryotrocha puerilis*, where the removal of a few segments can change the sex of the individual (Hartmann). Furthermore, the problem of sexual determination becomes clear only when we introduce the fundamental concept of *potential bisexuality*, according to which each organism is potentially of both sexes, but with one of them dominating the other. It is only thus that we explain the well known phenomenon of sex reversal after removal of a gonad in the hen or the toad. After taking out the gonad, the inhibited gonad of the opposite sex is able to develop.

We will limit ourselves to a few examples which have been studied sufficiently, so that we are able to draw certain conclusions. First of all, we will examine the well known case of *Bonellia viridis* and then we will consider the important work of Kuhn and Moewus devoted to sexuality in the unicellular alga, *Chlamydomonas eugametos*. After a brief examination of sexuality in mammals, we will discuss finally the relation between metabolism and sex determination.

### 1. Case of *Bonellia viridis*

We know that the female of this marine worm has the structure of a sac about 7 to 8 cm. long to which is attached a proboscis attaining a length of almost a meter. The male measures only a few millimeters and lives as a parasite in the intestine or in the uterus of the female. The fertilized eggs give rise to swimming larvae, which soon become attached to solid objects; before this, sexual differentiation is not yet established (Baltzer); if the larvae become attached to any ordinary support they develop into females, but if they fix themselves to the proboscis of a female they become males. When the larvae are placed in sea water containing a fragment of the proboscis, a large number of them transform into "intersexes," that is, they show characters intermediate between male and female.

These interesting observations show that the proboscis of the female exerts a masculinizing influence on swimming larvae which are as yet undifferentiated. Baltzer has observed that aqueous extracts of the proboscis also have this masculinizing effect. The active substance is resistant to boiling and wide variations in pH, while acetone extracts are on the contrary inactive (Nowinski); it would seem therefore that the proboscis contains a water soluble "hormonal factor." However, Herbst has shown that the change of the swim-

ming larvae in a masculine direction can be obtained by the addition of very simple reagents such as: hydrochloric acid, carbonic acid, traces of copper, magnesium, potassium. Thus, masculinization can be attained experimentally by a variety of agents. It is reasonable that the substance, as yet unknown, in the proboscis is the only one physiologically active.

Herbst believes that the inorganic agents which he has used modify sex through an effect on the respiratory activity of the larvae, a high respiratory rate being characteristic of the female. This is an hypothesis that would be easy to verify experimentally, and it would be interesting to know whether the intensity of respiration changes at the time when the sexes differentiate under various influences (proboscis, aqueous extract of proboscis, inorganic salts and acids). As Baltzer suggested, it would be very useful to study the action on the sex determination of *Bonellia*, of various substances specifically affecting respiration, for example, inhibitors such as cyanide, carbon monoxide, hydrogen sulfide, and activators like dinitrophenol, methylene blue, or pyocyanine. Furthermore, it would be wise to make a parallel study of the effects of these substances by a direct measure of metabolism.

This example illustrates how rudimentary is our knowledge in this field, since even the simplest experiments which would serve as a point of departure for a more thorough analysis have not yet been attempted. It is clear that the biochemical study of the classical case of *Bonellia* is only in its initial stages, and one hopes that the metabolism of the larvae of *Bonellia* will be the object of research in the near future.

It should be noted that, according to Baltzer, a small number of the larvae (5%) swim freely, do not attach to the proboscis, and still become males. Undoubtedly this is a matter of some individuals which have their sex determined genotypically. Thus it is clearly seen in the case of *Bonellia* that sex may result from a combination of genotypic and phenotypic factors—with a preponderance of the latter. From the chemical viewpoint, we see once more that very different agents can influence sex determination and stimulate the development of intersexes and even the reversal of sex. There is no reason to believe that these substances exercise their primary action on cellular oxidations and, at present, this concept must be regarded as purely hypothetical.

## 2. Sex Determination and the Fusion of Gametes in *Chlamydomonas eugametos*

The collaboration of the botanist Moewus and the biochemist Kuhn has been amazingly productive as a result of their studies of the chemical substances responsible for determination of sex and for the attraction of the gametes in a unicellular alga, *Chlamydomonas eugametos*. Their work has led to surprising conclusions regarding the chemical nature of the substances, the low concentration at which they act, and the relation of their appearance to the activity of certain genes. Because of the impact of such claims on biological thought, it would be interesting if these experiments were repeated and confirmed in other laboratories. It should be remembered that certain early results of Moewus were criticized severely by Philip and

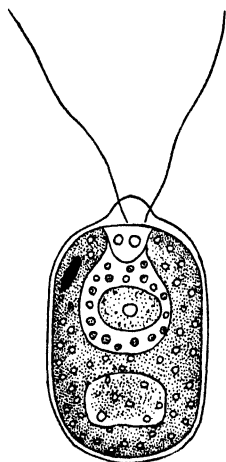


Fig. 8. Semidiagrammatic drawing of *Chlamydomonas* (after Massart).

Haldane. However, this was a matter concerning another field and it does not appear that these criticisms affect the biochemical work of Kuhn and Moewus (see Hämmerling for the details of this controversy). Sonneborn remarks that a certain amount of caution must be exercised in evaluating the results of Kuhn and Moewus as long as they have not been independently confirmed.

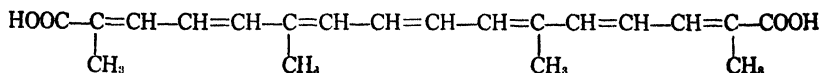
As seen in Figure 8, *Chlamydomonas* contains a nucleus, some chloroplasts (chlorophyll), a pyrenoid body that gives rise to starch grains, a pigment spot (stigma), some contractile vacuoles and two long flagella. This organism can reproduce itself either by simple

division (asexual type) or by conjugation of gametes. When it is cultured on agar, it is nonmotile and lacking in flagella and can reproduce only by division. If the cells are placed in a nutrient liquid medium and illuminated, two flagella appear and the cells become motile. A more prolonged illumination induces conjugation of the cells, and male and female stocks are found. However, these are isogametes having the same structure. When the gametes of the two sexes are mixed, they conjugate immediately and form zygotes. It should be pointed out that it is not possible in complete isogamy to speak of male and female sexes, but, more correctly, "+ sex" and "- sex." It is, however, possible to cross the gametes of the isogamous alga with those of an anisogamous variety and thus determine the true sex of the gametes.

In particular, Kuhn and Moewus have studied two forms: *simplex*, which is dioecious, that is the sexes ( $\sigma$  and  $\varphi$ ) are separate, and *synoïca*, which is bisexual ( $\sigma\varphi$ ). The first form lends itself well to the study of factors that govern the attraction of gametes of opposite sexes, while the second constitutes excellent material for the study of chemical agents that transform the bisexual cell ( $\sigma\varphi$ ) into male ( $\sigma$ ) or female ( $\varphi$ ).

Let us first examine the question of the appearance of the flagella and the fusion of the gametes of *Chlamydomonas eugametos* var. *simplex*. The flagella appear only when the cells are placed in a liquid medium and are subjected to the action of light. The latter is necessary for the development of the flagella, but certain sugars in the presence of oxygen can be substituted. Glucose, maltose, lactose, and sucrose render the cells motile in the dark. The most active sugar is *gentiobiose*, a glucose-base disaccharide ( $\beta$ -glucosido  $< 1, 5 >$ -6-glucose  $< 1, 5 >$ ). There is still another procedure for making the gametes motile in the dark. If a suspension of *Chlamydomonas* is illuminated and filtered, the filtrate causes flagella to form and makes the cells motile even in the dark and without oxygen. This fundamental experiment shows that cells rendered motile through the effect of light excrete into the external medium a substance that stimulates motility in the gametes. This particular substance has been concentrated and it shows the absorption spectrum of certain carotenoid pigments present in saffron. It is found only in traces in the filtrate—about one milligram of pigment in one thousand liters. The physical and chemical properties of this substance are

similar to those of a carotenoid isolated from saffron, *crocin*, which is an ester of crocetin and of gentiobiose. The formula for  $\alpha$ -crocetin is:



Kuhn, Moewus, and Jerchel have tested the activity of crocin extracted from saffron on *Chlamydomonas* and, at a dilution of 1:  $25 \times 10^{13}$ , this substance makes the gametes motile in the dark. Repeated experiments have shown that 5 molecules of crocin are sufficient to make 4 gametes motile. Thus we come to the conclusion that one gamete of *Chlamydomonas* needs only 1.25 molecules of crocin to become motile! The biological activity of this substance is really enormous.

Recently, Moewus (1943) again studied the question of the appearance of the flagella and of motility. The results of some very careful experiments show that the flagella can develop in the dark if glucose, crocin, or a filtrate of illuminated cells are added. However, these flagella will not begin to beat unless a supplementary fraction, as yet unknown, is added. This factor is found in the filtrate and as an impurity in certain preparations of crocin.

Thus crocin is the substance causing the flagella to develop in the dark and a second factor is necessary to make them functional; but this alone is not sufficient to insure conjugation and one can predict that the attraction of the gametes will depend on other chemical compounds. As a matter of fact, illumination by red, yellow, or green light stimulates motility without copulation, which takes place only with blue or violet light. This fact leads us to believe that a second photochemical reaction results in the production of substances that draw the gametes toward each other. The isolation of the active substance has been carried out by the following experiment. A suspension of *Chlamydomonas* is illuminated by a red light and then filtered. As we already know, this filtrate renders the cells motile without causing conjugation. Now, when this "red" filtrate is illuminated with blue light for varying times and its biological activity is examined after 25 minutes, it is noted that the female cells are capable of conjugating. It is necessary to prolong the exposure time to blue light to 75 minutes in order to change the "red" filtrate so that it renders the male gametes capable of conjugating. If the

treatment is continued the filtrate finally loses all its activity. Kuhn, Moewus, and Jerchel soon found that the "red" filtrate could be replaced by a photosensitive substance which Kuhn had previously isolated from safron. It is the *cis*-dimethyl ester of crocetin which is transformed rapidly into the *trans* isomer by the action of blue light.

A detailed analysis of the phenomenon has shown that the substance enabling the female gametes to conjugate is a mixture of three parts of the *cis* ester with one part of the *trans* ester of dimethylcrocetine. The male gametes are able to conjugate when they are treated with a mixture of three parts of the *trans* ester and one part of the *cis* form. These substances are specific to a very high degree and act in the tremendous dilution of 1 part in 33 million.

These biochemical observations explain remarkably well the earlier observations of Hartmann on the "relative sexuality" of the alga *Ectocarpus siliculosus*. In this form the gametes are not *absolutely* male or female since it is found that some gametes behave like females in relation to certain gametes but like males in relation to others. A gamete that is a "weak" female can conjugate with a "strong" female, and a "weak" male behaves as a female in relation to a "strong" male. Hartmann arrived at the conclusion that sexuality in the algae must be determined on a quantitative basis and not on qualitative differences. The work of Kuhn and Moewus (1940) confirmed this opinion in a striking manner for they showed that in order to have conjugation between two cells it was sufficient to have a difference of 20% in the composition of the mixture of *cis* and *trans* esters which the two gametes secrete. In *Chlamydomonas* one distinguishes in each sex four types of gametes of graded "strength" which differ according to the proportions between the two esters. The characteristic values for the ratio *cis/trans* for the various types of gametes are:

	♀ <sup>4</sup>	♀ <sup>3</sup>	♀ <sup>2</sup>	♀ <sup>1</sup>	♂ <sup>1</sup>	♂ <sup>2</sup>	♂ <sup>3</sup>	♂ <sup>4</sup>
<i>cis</i>	95	85	75	65	35	25	15	5
<i>trans</i>	5	15	25	35	65	75	85	95

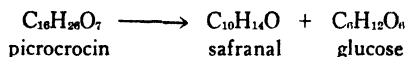
The proportions 75/25 and 25/75 indicated above thus refer only to average females and males.

The various substances just considered stimulate conjugation and are the fertilization substances which as we shall see also, have

their counterparts in the metazoa. They are termed *gamones* (andro- and gynogamones). It is also known that the secondary sexual characteristics of vertebrates are due to chemical substances, the *hormones*. There is a third class of sex substances, the *termones*, which determine sex itself in organisms with a "mixed" sexuality.

This brings us to the work carried out on the *synoïca* form, in which the sexes are not separated as in the dioecious species we have just considered. The two algae, *synoïca* and *simplex* are very close to each other. Moewus first noticed that if one adds to the sexually indifferent cells ♂ a filtrate of the female gametes, all the cells become females; conversely they all transform into males if treated with a filtrate from male gametes. It is this experiment which stimulated Kuhn, Moewus, and Wendt to investigate the chemical nature of these "termones" present in the filtrates (gyno- and androtermones). The concentration of the feminizing and masculinizing substances in the filtrates is very weak (about 1 mg/1000 liters). The androtermone is volatile in steam and soluble in ether, while the gynotermone has neither of these properties. Kuhn and co-workers noted further that heating the gynotermone with sulfuric acid or with dilute barium hydroxide transformed it into androtermone.

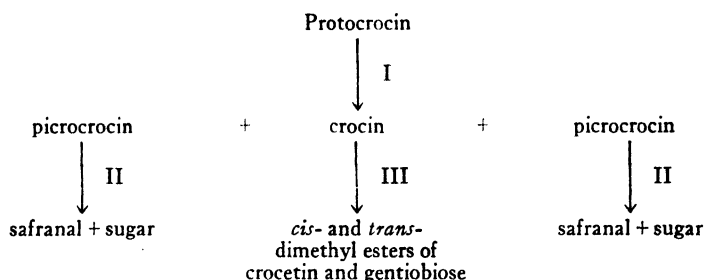
These properties made it seem reasonable that androtermone and *safranal*, were identical; the latter substance is responsible for the odor of saffron. The gynotermone would be identical with *picrocrocin*, the bitter substance of saffron. Picrocrocin is in reality a glucoside of glucose and safranal and its hydrolysis can be formulated as follows:



It is sufficient to add only 0.02 gamma of picrocrocin to the cells to transform them into females capable of conjugating with the male gametes, and if the picrocrocin is hydrolyzed so as to transform it into safranal it becomes a masculinizing substance. Nevertheless, these substances are less active than the gamones. While only about 10 molecules of safranal are needed to masculinize the ♂ cells, a concentration of  $10^6$  molecules per cell is necessary in the case of picrocrocin. This suggests that these substances are not the true termones, but simply similar compounds. Further, Kuhn, Moewus, and Wendt have observed that picrocrocin is about 1000 times less

active than the true gynotermone present in the filtrate. We will see later that the real nature of the termones is now known.

The scheme on page 36, taken from Kuhn, Moewus and Wendt, gives at a glance the chemical composition of the substances under discussion. Their relationships would be established in the following manner: a carotenoid, as yet unknown, protocrocin (1), would be transformed into crocin (2) and into picrocrocin (5); crocin would give rise to the dimethyl esters of crocetin (3) and of gentiobiose (4), while picrocrocin would be transformed into safranal (6) and sugar.



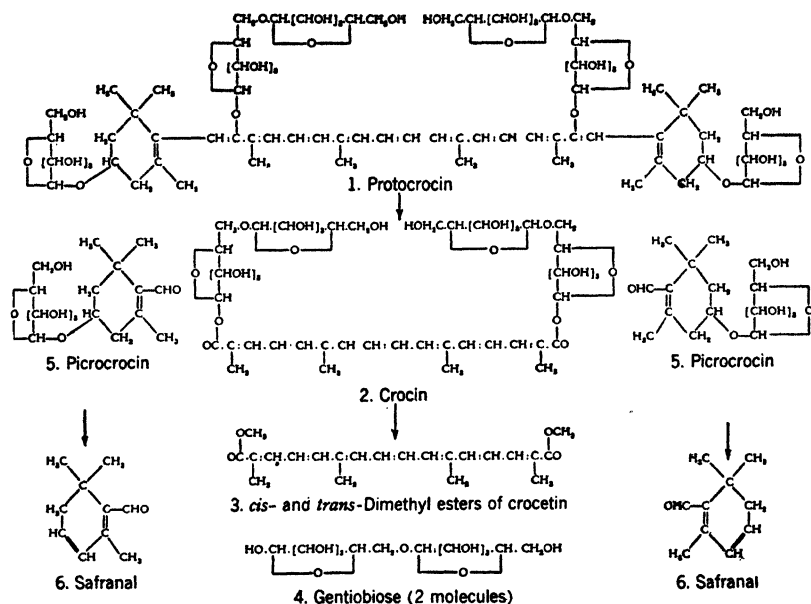
The various reactions which appear in this scheme are controlled by some *genes*, that is to say by chromosomal factors that determine hereditary characters. Moewus has stated that, if one submits *Chlamydomonas* to a high temperature, mutants can be obtained which secrete neither crocin nor the two termones. These mutants are rare, comprising only 14 gametes in more than 60,000 studied, and they are characterized by their immobility, even in light, and by their inability to reproduce sexually.

As we cannot dissociate the production of termones from that of crocin, it is probable that the secretion of these different substances is controlled by the same gene (gene Mot, from *motus*—movement). This gene would regulate the formation of crocin and of the termones at the expense of a single precursor. Thus it would influence reaction I:



Reaction II of the scheme of Kuhn, Moewus, and Wendt (picrocrocin  $\rightarrow$  safranal + sugar) has also been analyzed from the genetic point of view. This transformation is possible only in the male





gametes which possess a gene M that insures the secretion of the proper proportions of the mixture of *cis* and *trans* isomers. A second gene  $M_D$  is responsible for the action of male cells on the determination of the sex of  $\sigma^7$  cultures and induces them to synthesize and secrete the androtermone. Kuhn and Moewus (1940) have found that the *male* cells and *only these cells* contain an enzyme that hydrolyzes picrocrocin or the filtrate from female cells into an androtermone (safranal) and a sugar. This enzyme can be detected only in a very fine brei of the cells. If this brei is centrifuged, the centrifugate alone is active and the supernatant liquid is completely lacking in the enzyme, which thus is seen to be bound to a cellular structure. Since the enzyme appears to be absent in the flagella and cellular membranes, we can reasonably assume that it is present in the *nuclei*. This enzyme shows other curious properties, being very sensitive to temperature with an optimum around 26° C. The pH optimum is 7 and activity decreases on either side of this value. Finally, this enzyme either has very low activity or is present in exceptionally low amounts, for we see that one *Chlamydomonas* cell under most favorable conditions hydrolyzes one molecule of picrocrocin per second while a yeast cell splits  $10^{10}$  molecules of sac-

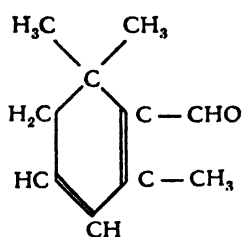
charose in the same time. Furthermore, this enzyme can be detected only by the biological properties of the androtermone which it forms and there is no physical or chemical method sensitive enough to demonstrate it (if one followed the enzymatic reaction polarimetrically, it would take 30,000 years to obtain a 50% hydrolysis of picrocrocin). This enzyme can attack two different substrates, picrocrocin and the true gynotermone present in the filtrate of female gametes. The latter, which perhaps differs in the nature of the sugar of the glucoside, is attacked 4-6 times faster than picrocrocin.

Kuhn has stated that the enzyme that hydrolyzes picrocrocin bears a very close relation to the gene  $M_D$  and that many of the facts argue for an *identity* of the gene and the enzyme. This latter, like genes, is a thermolabile substance of high molecular weight; it is present in the cell in very small amounts and is directly bound to a cell structure, perhaps the nucleus; and finally, it is found exclusively in genetically male cells. The gene  $M_D$  would thus be an enzyme hydrolyzing the glucoside of a terpene, although there may still be found some unknown compound between the gene, and the enzyme. Thus, for the first time, experimental analysis has made a real contribution to the problem of the chemical structure of the genes and confirms the hypothesis, so frequently put forth, since Goldschmidt, of the enzymatic nature of the gene.

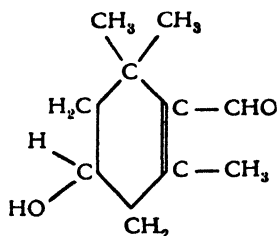
There is, finally, a third chemical reaction controlled by genes in *Chlamydomonas*. This is the transformation of crocin into the dimethyl esters of crocetin (reaction III in the scheme of Kuhn and Moewus). The gene causing it ("gathe") is responsible for the secretion of the gamones and regulates the temperature limits within which they are produced. For example, certain northern forms (*C. rigophilus*) can conjugate only between 1° and 16°, while *C. typica* conjugates between 4° and 36°. The elimination of gamones in the definite proportions indispensable for conjugation takes place only within the fixed temperature limits. This is because the enzymes which esterify crocin act only between these same temperatures. These enzymes are found in the two types of gametes (♂ and ♀) in an inverse ratio to the enzymes that hydrolyze picrocrocin. In addition, there appear to be two distinct enzymes which esterify the *cis* and *trans*-crocine, respectively. These are found in breis of the gametes and the temperature optimum is 27° for *C. typica* and 12° for *C. rigophilus*. The pH optimum is very sharp and in the

neighborhood of 7. In one second a cell esterifies about 5 molecules of *cis*-crocin, so that we may suppose that the enzyme is present in traces. If extracts are prepared from the monoecious race (*Chlamydomonas eugametos* var. *synoica*) they retain the ability for esterifying the *cis*-crocin. In this case the reaction is activated by traces of picrocrocin, while it is inhibited by small quantities of safranal. This explains how the *cis/trans* ratio can be modified, for the formation of the *cis*-dimethyl ester of crocetin (gynogamone) is activated by the picrocrocin (gynotermone) and is inhibited by safranal (androtermone). The termones, by activating or inhibiting the esterifying enzymes, assure the production of the proper proportions of gamones.

The description above has been taken chiefly from reviews published by Moewus (1939, 1941) and by Kuhn (1940). Since then certain refinements have been added so that now Kuhn and Löw have isolated an *androtermone* much more active than safranal. This is an hydroxyaldehyde, very similar to 4-hydroxy-2,6,6-trimethyl- $\Delta'$ -tetrahydrobenzaldehyde. Only 1.3 molecules is sufficient to transform a cell into a male.



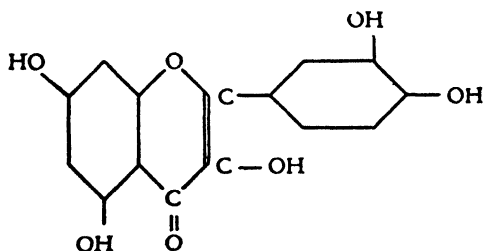
safranal



androtermone

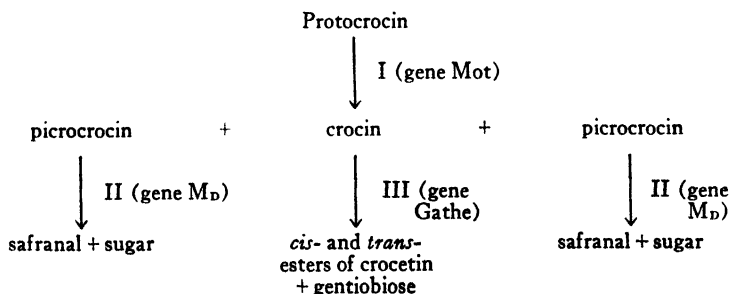
More recently, Kuhn, Löw, and Moewus have discovered some new details concerning the nature of the *gynotermone*. They found that cells become males and conjugate with female gametes when traces of boric acid are added ( $1/10^{10}$ ). Thus 500,000 molecules of boric acid are sufficient to make boron behave like an "inorganic androtermone." This fact has led to the isolation of the *gynotermone* which forms a part of a glucoside present in one species of crocus. The *gynotermone* is the aglycon of the glucoside (nucleus to which two sugar molecules are attached). This substance is 100,000 times more active than picrocrocin and acts in the proportion of 10

molecules to one cell. As a matter of fact, the picrocrocin first studied contains the gynotermone as an impurity and it would appear that the picrocrocin has no other physiological role than to be the mother substance of the hydroxyaldehyde (androtermone). The aglycon forms a complex with boric acid and this fact clarifies the action of the latter. Thus the cells eliminate both the aglycon (gynotermone) and the hydroxyaldehyde (androtermone). An excess of the latter stimulates masculinization and the addition of boric acid, by blocking the gynotermone, leaves an excess of androtermone. The chemical nature of this aglycon is not yet definitely established. It may be a methyl ester of quercetin.



quercetin

Kuhn and Moewus have not yet announced, to our knowledge, the necessary modifications of their diagram in the light of these new facts. Recalling their original scheme:



it is evident that the substitution of the hydroxyaldehyde for safranal as the androgamone does not essentially change the scheme. Picrocrocin remains the precursor of androtermone, but it is deprived of activity as a gynotermone.

The scheme of Kuhn and Moewus explains three facts: that the gynotermone can not appear independently of the gamones; that male cells transform picrocrocin into androtermone; that the cells can transform crocin into gamones. However we now see that the gynotermone differs considerably from the carotenoids on a chemical basis. Reaction I must then necessarily be more complicated than is indicated by the diagram. Perhaps the mother substance may be more complex than the protocrocin, and may contain the gynotermone in its molecule. It would be desirable for the analysis to be continued, and for the remaining obscure points be cleared up. It seems, however, that the new facts do not radically alter the ideas formulated by Kuhn and Moewus. Regarding the gene  $M_D$  specifically, the authors have taken care to make some tests on filtrates which show more activity than picrocrocin.

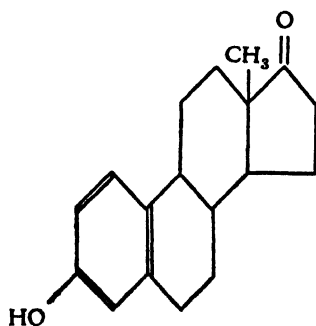
Regardless of the eventual changes which may be necessary in the future, several fundamental findings appear to be well established: Sex is determined in *Chlamydomonas* by chemical substances that have been identified (andro- and gynotermones); they act in very low concentrations (of the order of one molecule per cell). Conjugation of the gametes is insured by the secretion of gamones in definite proportions, and it is the ratio between the *cis* and *trans* isomers which is responsible for the relative sexuality theory of Hartmann. The gamones, like the termones, are active in traces. Finally, there are enzymes present in the cell which catalyze the reactions controlled by genes and there are good reasons for believing that these enzymes are identical with the genes.

### 3. Determination of Sex in the Vertebrates

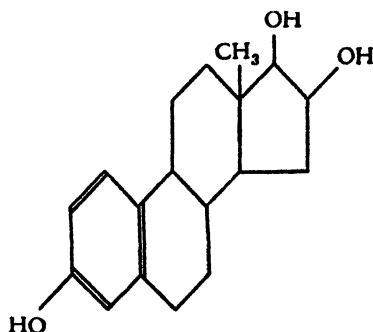
Let us leave the protozoa and go on to the higher organisms, much more complex from both the anatomical and the physiological point of view. We will see, however, that the determination of sex is fundamentally the same, that is to say, the change toward a definite sex is stimulated here too by chemical substances.

We shall be brief in discussing this point since the question of sex determination has recently been treated in a clear and complete fashion by Caullery (*Organisme et Sexualité*, 1942) and by Dantchakoff (1941). A discussion of the problem together with an important bibliography is found in an interesting monograph by A. Raynaud. We will limit ourselves here to a simple outline of the

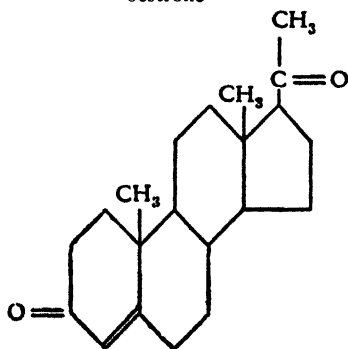
subject. We know that secondary sexual characteristics (wattles, spurs, comb, crowing, for example) are determined by the *sex hormones* secreted by the interstitial cells of the gonads (Ancel and Bouin). The biochemical research of Allen and Doisy, Butenandt, Ruzicka, Laqueur, etc. have led to the isolation of these hormones. They are all derivatives of the *sterols* and thus belong, like the carotenoids, to a major class of the lipids having, however, a polycyclic



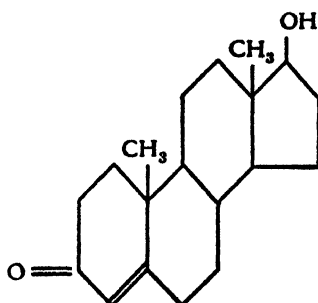
oestrone



oestriol



progesterone



testosterone

structure rather than a straight hydrocarbon chain. The most important ovarian hormones are *folliculin* (oestrone), *oestriol* or *cestradiol*, this last substance being physiologically more active. Equilin and equilin, of similar chemical composition have been isolated from the mare. These substances are found in the follicles containing the eggs but are also found in the male. The urine of the stallion is particularly rich in oestrone. The corpus luteum, which is the

ruptured follicle after the escape of the egg, contains *progesterone*, which modifies the structure of the uterine mucosa. Progesterone has been found in abundance only in the ovary. Finally, there are the male hormones such as *androsterone* found in human male urine and in nonpregnant women, and *testosterone*, more active, which comes from the testis itself and seems to be the physiologically active hormone. The formulae of these hormones appear on page 41.

About 1936, experiments were begun to find out whether the male and female hormones would exercise any action on the determination of the *primary* sex characteristics, that is, on the development of the gonads themselves. The experiments consisted of applying the hormones to embryos of such age that the sex glands were not yet differentiated. The question has been attacked almost simultaneously by Wolff and Ginglinger, by Willier and his collaborators and by V. Dantchakoff. Willier had noted as early as 1934 that the genetic males of chick embryos were feminized by female sex hormone. This change was rarely complete and intersexes were usually obtained.

Wolff and Ginglinger, as well as Dantchakoff, have shown that male embryos treated with oestrone are drastically modified, forming intersexes which can cursorily pass as females. These alterations however, are only temporary and the chicks end up by regaining their genetically determined sex. The action of male hormones is more complex and Willier as well as Wolff and Ginglinger, showed that testosterone stimulates a tendency toward intersexuality in both sexes. In Dantchakoff's experiments this hormone is practically inactive.

The influence of hormones on the determination of sex has been found in other species of vertebrates. Oestradiol reverses the sex of genetically male tadpoles (Gallien) and this author has recently announced a strange situation wherein dehydrofolliculin injected as an oily solution is feminizing while the same substance added as an aqueous solution in the aquarium water is masculinizing. In the amphibians Gallien obtains a persistent masculinizing effect with the male hormone. In reptiles, Dantchakoff and Kehl have found the same results as in the chick, namely: folliculin is feminizing while the male hormone leads to the formation of intersexes regardless of the genetic sex of the embryos. In the guinea pig, Dantchakoff obtained a masculinization of the young embryo by

means of male hormone. The injection of oestradiol into the pregnant female generally brings about the death of the embryo and abortion. However, Raynaud has noted a tendency toward intersexuality in genetically male embryos treated with female hormone.

At first, it would seem that these hormones are the agents which determine sex in vertebrates, but it is seen that these substances have transient effects that are often paradoxical; thus, for example, Foote observed that testosterone gave a feminizing reaction in the case of *Amblystoma* larvae. In the face of facts of this sort, Witschi suggested another explanation. The gonads in the tadpole are composed of two distinct parts, cortical region and medullary region, in close proximity. The former induces ovarian differentiation, while the second stimulates testicular differentiation. These gonadal factors added to the genetic constitution and environmental factors regulate the determination of sex. In the vertebrates, it has been established that the chromosomes are not the only factors operating, for, if reversal of sex occurs in the young hen as a result of removal of the left ovary, the testis which develops at the expense of the right gonad contains the number of chromosomes characteristic of the female. Thus the genes can only act by modifying the equilibrium existing between the cortex and the medulla. The "inducing" substances in the young gonad can not be, according to Witschi, the sex hormones, for these do not appear until a much later stage in development. Witschi suggests that the cortex and the medulla contain two hormones which always *antagonize* each other, a fact that is not general as we have seen in the case of the sex hormones. He rejects the hypothesis formulated by Dantchakoff, according to which the heterochromosomes form the sex hormones directly and thus determine sex. The sex hormones of the adult act only as modifiers of the external environment of the gonad and alter the normal mechanism of sex determination, producing abnormalities. The hormones present in the cortex and the medulla, which are responsible for the differentiation of the gonads, have nothing in common with the steroid hormones, and to all appearances they behave more like proteins.

We see that, although the differentiation of sex in vertebrates is brought about by chemical substances, the situation is not entirely clear. The steroid hormones certainly lack specificity in the kind of reactions they stimulate. It would be premature to maintain that they play no role whatever in sexual determination, conforming to



Witschi's theory. But Witschi is correct in placing the problem in the field of chemical embryology. There are active chemical substances present in the young gonads which are truly important and it is the elucidation of their nature on which future efforts should concentrate.

#### 4. Sex Determination and Respiratory Metabolism

One last aspect of the problem of sex determination remains to be examined—whether the rate of respiratory metabolism constitutes an important, if not decisive, factor in this process. We have seen that well defined chemical substances are responsible for sex differentiation in the vertebrates as well as in the protozoa. This conclusion does not exclude the possibility that these substances are dependent for their formation on variations in the rate of metabolism of the two sexes. The “metabolic” theory of sex determination has been suggested repeatedly. Presented in 1889 by Geddes and Thompson, it has been especially developed by Riddle and Joyet-Lavergne. The latter has summarized, in a publication in 1931, all the observations made on the subject up to that date. Numerous works are cited which establish the reality of metabolic differences between *adult* individuals of the two sexes. We believe, however that these results are only of limited value, for what we really want to know is whether these metabolic differences are present *before* the time when the gonads differentiate into ovary or testis or whether they occur at this same time. That there are differences in the rate of metabolism of male and female adults is not surprising, considering the differences in the anatomy and physiology of the two sexes. This is a case of a result of the existence of two sexes rather than a cause.

We will consider then only the results of those investigators who have recognized the importance of the above objection. Riddle has made several experiments with this in mind, too indirect, unfortunately, to be convincing. Struck by the fact that the production of females in the fowl increases in summer, he determined the “internal energy” of the eggs in a bomb calorimeter and found it to be higher in summer. In addition to the fact that this does not prove that all the eggs with a high potential energy develop into females, one may question whether the method used permits any deductions regarding the rate of metabolism. Riddle has not measured the latter directly, and supposes that the increase in potential energy (*higher storage*)

in the female is an index of a lower metabolism. This potential would be lower in the male because the more intense oxidations prevent the storage of reserves. Riddle has also observed that the mortality of male chick embryos is greater than that of females when they are subjected to a low oxygen tension or to a relatively low temperature. These environmental changes would lower the metabolism, and the males, in which the metabolism would be highest, would suffer more. It is well to recall at this point that the investigators who have measured the metabolism of the chick (see especially Mitchell, Card, and Haines) have found no statistically significant differences between males and females.

To these indirect observations is opposed the careful and recent work of I. A. Wills, a student of Witschi. Witschi has shown that reversal of sex occurs in the frog when the surrounding temperature is raised. Riddle has deduced from this that the higher temperature accelerates metabolism and, conforming to his theory, stimulates the formation of males. Wills measured the oxygen consumption by Warburg's method in three species of urodeles, and two species of anurans from the beginning of development, at fertilization, to the adult stage. He concluded that there is no difference in the rate of respiration between males and females at the time when the gonads differentiate. It is only much later that an increased metabolism is observed in the male. Thus Wills' observations are in direct opposition to Riddle's hypothesis and show that the explanation proposed by the latter can not be accepted, at least for the amphibian embryo.

Joyet-Lavergne has succeeded in attacking this problem using simpler and thus more favorable material. He worked with the sporozoa (gregarines) particularly, but the results have been extended to several plants (algae, fungi, horsetails, phanerogams gametophytes). Joyet-Lavergne determined mainly the  $rH_2$  (oxidation-reduction potential) of male and female gametes. His conclusion is that the  $rH_2$  of the female cells is lower than that of males, which signifies that the reducing power of the former is greater. Unfortunately, the techniques of Joyet-Lavergne are subject to grave reservations, for, in general, he used vital dyes which present the difficulty of being reduced only at the granules of the cells to which they become adsorbed. The use of such methods has been criticized by Chambers and his collaborators. Joyet-Lavergne attempted to determine glutathione by the nitroprusside reaction because of its re-

ducing power, but this reaction reveals all the substances which contain the —SH group and it is necessary to take into account those groups which are liberated by the denaturation of the proteins. Joyet-Lavergne tried also to demonstrate vitamin A by the Carr and Price reaction with antimony trichloride. This technique is far from specific for many of the carotenoids give a blue color with the reagent. Because of the uncertainty of the methods it is not profitable to discuss the ideas of this author concerning the role of the chondriome and the nucleolus in oxidations, for they also have a doubtful basis and have been criticized severely by Rey and Wurmser. Regarding the conclusion of Joyet-Lavergne on sexuality, we believe that there are real differences between the two sexes but that it is not possible to say precisely what these differences are. We can not use evidence involving metabolism and oxidation if we do not use for their determination the quantitative methods at our disposal. We have often seen how dangerous it is in chemical embryology to attack these questions of cellular physiology by means of methods which are deceptively simple and which have doubtful significance. They are open to many false interpretations, and the conclusions drawn from them are subject to question. In turning to the simpler organisms, especially those that, like the gregarines, show isogamy in order to determine significant differences in metabolism, Joyet-Lavergne chose an excellent point of departure. Unfortunately, however, he selected inappropriate methods so that the work should be repeated with more modern and precise procedures.

In speaking of the observations of Joyet-Lavergne on the gregarines, it is interesting that the more recent work of Göhre has led to somewhat contradictory results. He was unable to find any differences between male and female cells with regard to pH, glutathione content, vitamin C and peroxidases. However the anterior region of the female organism gave a higher  $rH_2$  and showed the indophenol oxidase reaction selectively and this part also appears to be richer in iron. Nevertheless, one can not draw conclusions regarding the determination of sex because this anterior region of the gregarine becomes fixed to the host which it parasitizes and it is this region through which food it taken in. Göhre believes that sex determination in the gregarines is very early and genotypic in nature.

The results of Wills on amphibia have been confirmed in *Drosophila*. Poulson has observed that at the time of pupation, the

respiration of males is higher than that of females but that there is no difference between the sexes during the first few hours after fertilization (Boell and Poulson). Furthermore, the introduction of an extra X or Y chromosome does not modify respiratory metabolism. These very interesting observations prove that the chromosome complex, notably the balance between the heterochromosomes and the autosomes, does not control metabolism. Thus genotypic determination of sex is not primarily a modification of the respiratory metabolism.

Although the situation appears unfavorable for a "metabolic" theory of sex determination, there should certainly be a repetition of the experiments on simpler isogametic organisms. For example, a study of the respiratory metabolism in *Chlamydomonas*, where sexuality has been so completely analyzed by Kuhn and Moewus, still remains to be done. In addition to measurements of oxygen consumption it would be necessary to investigate the effects of substances which stimulate and inhibit respiration on the production of gamones and termones. It will then be possible to take a definite stand for or against the metabolic theory of sex determination. A first step in this direction appears to have been taken by Boell and Woodruff. Sonneborn and Jennings have shown that in *Paramecium* there are different mating types during conjugation. The individuals belonging to types I and II agglutinate each other and form pairs when mixed under certain conditions. Boell and Woodruff studied the oxygen consumption of these types using the Cartesian diver. In the forms in which agglutination is followed by conjugation, they obtained a value of  $0.25 \times 10^{-3} \text{ mm}^3$  for type I and of  $0.28 \times 10^{-3} \text{ mm}^3$  for oxygen per individual per hour for type II. These differences, though they may be very small, are statistically valid, but may be apparent only because the individuals of type II are really a little larger than those of type I. Organisms which do not agglutinate or conjugate have a much higher respiration ( $0.43$  and  $0.48 \times 10^{-3} \text{ mm}^3$  of oxygen for types I and II, respectively). When agglutination occurs without the formation of pairs, it was found that one of the partners had a low respiration ( $0.25 - 0.28 \times 10^{-3} \text{ mm}^3$  of  $\text{O}_2$ ) while the other member showed a high oxygen consumption (about  $0.45 \times 10^{-3} \text{ mm}^3$ ). There is thus an inverse relationship between the tendency to conjugate and metabolism; this is not surprising, since conjugation in *Paramecium* is frequent only when the individuals are

poorly nourished. If the individuals that form pairs should really be considered as possessing different sex, it would follow that there is but a very small difference (12%) in metabolism between the members of the two sexes.

### 5. Conclusions

The recent biochemical investigations have rapidly furthered our knowledge of sex determination in *Chlamydomonas* and in the higher vertebrates, and emphasis should be put on the striking parallel between the biological and chemical findings. Biologists have slowly given up the idea of absolute sex, and now consider all individuals as potentially bisexual. The biochemists have shown that sex determination can be affected by very small modifications in the composition of certain substances (*cis* and *trans* isomers, degree of saturation of the sterols). In *Chlamydomonas*, as in the vertebrates, it is probable that the different sexual compounds are formed when metabolism is the same in both sexes. In the case of *Chlamydomonas*, masculinization is conditioned by a gene  $M_D$  which consists of an enzyme (or functions as an intermediate substance for an enzyme) liberating the androtermone from an inactive precursor (protocrocin or similar compound). The notion of "relative sexuality" (Hartmann) is confirmed in a striking fashion by the demonstration that the copulation of the gametes is controlled by the excretion of a mixture of two gamones in definite proportions. The fact that the sex hormones of vertebrates show little specificity and that they are found in both sexes agrees well with the notion of bisexual potentiality.

There is no longer any doubt that sex is determined by chemical substances or by the ratio of concentration of special chemical substances. The reactions which lead to their formation in definite quantities from precursors must obviously be catalyzed by enzymes that may be identified, in part or in whole, with genes. We can see without difficulty that the reactions could also be modified by changes in the external environment such that the optima for these enzymes are distinctly different (in *Chlamydomonas*, at any rate). We can foresee from the above the principles of double determination of sex—genotypic and phenotypic. Genes produce directly or indirectly the sexual substances by means of reactions which can be modified by external conditions by either stimulation or inhibition. At present,

we are merely utilizing a hypothesis based on the results of Kuhn and Moewus. The biochemical analysis in these forms, in which sex determination is in some cases genotypic in others phenotypic, will allow us to bring the facts more closely together. Its effectiveness has already been shown so that we may reasonably hope that in the future it will reveal new facts in the realm of sex determination.

The role of respiratory metabolism in this phenomenon remains obscure. At present there are no indications in favor of early fundamental differences of metabolism in the *embryos* of different sexes and the few facts at our disposal run contrary to the theories of Riddle and Joyet-Lavergne. This conclusion, we shall see, is in harmony with other work, for in several connections we are inclined to regard respiratory metabolism as a relatively unimportant morphogenetic agent and that it plays only an accessory role compared to specific chemical substances.



## CHAPTER III

# Formation of the Gametes

We will examine first oögenesis and then spermatogenesis; we know that the development of the gametes takes place as follows: after a period of multiplication of the stem cells (gonia), a growth in size occurs; this latter remains moderate in the case of the spermatocyte but is considerable in the oöcyte. These primary cells undergo two successive maturation divisions: the first reduces the number of chromosomes by one half (meiosis or reduction division) and gives rise to secondary oöcytes or spermatocytes; the second division is an ordinary mitosis and leads to the formation of oötidis or spermatids. The latter then transform into spermatozooids (spermiogenesis). Let us recall that in the oöcyte the maturation divisions are very unequal: only a small amount of cytoplasm is separated from the egg and the "polar bodies" eliminated play no role in subsequent development.

### 1. Oögenesis

The large increase in size of the oöcyte arouses great biochemical interest: in fact it is a period in which the syntheses are particularly intense and in which anabolism is more important than catabolism; the oöcyte accumulates reserve substances that insure later development. Naturally, these reserves will be proteins, lipids, and carbohydrates: the first form the vitelline granules in small eggs, the vitelline particles in forms rich in reserve substances (deutoplasm); these particles are often lipoprotein in nature and frequently show anisotropy which is similar to crystals. They generally contain phosphorus (phosphoproteins); for more details we refer to Needham's book, in which a number of analyses of the yolk of different species will be found. The fats appear in the form of droplets staining with



Sudan III; as a result of their low specific gravity they accumulate at the centripetal pole when the egg is centrifuged. The recent works of Holtfreter have established that the fats in the frog's egg are not found in the form of free lipid droplets: they are really *lipochondria*, that is, granules in which the lipids are surrounded by a protein envelope. The denaturation of these proteins by various agents stimulates the transformation of the lipochondria into lipid droplets; such a transformation occurs during cytolysis and in normal development.

As to the yolk, it is formed of large particles, which the centrifuge throws to the centrifugal pole; Holtfreter has recently devoted a careful study to the reactions of the vitelline particles to various agents (acids, alkalis, salts, etc.). There is often a "vitelline gradient" in the eggs, the size of the particles growing progressively larger from the animal to the vegetal pole. This gradient becomes very apparent (J. Brachet) when the frog's oöcytes are subjected to the cytochemical reaction of Serra for the determination of arginine: the amount of arginine in the granules increases progressively going from the animal toward the vegetal pole. As to glycogen it appears and stains in fixed preparations as granules or flakes scattered throughout the oöcyte.

Between these cytoplasmic inclusions is found the "true protoplasm" or hyaloplasm, which is demonstrated by basic dyes. In centrifuged eggs it forms a layer situated between the lipid cap and the vitelline layer; it is significant that one finds in centrifuged oöcytes a thin, basophilic cortical layer which persists and is not displaced by centrifugation. The nucleus (germinal vesicle) is voluminous; a very clear membrane separates it from the cytoplasm; this membrane is birefringent (Schmidt) because of its structure: it consists of concentric protein lamellae or folioles. The germinal vesicle contains an abundant nuclear sap, and one or more large nucleoli; the chromosomes are greatly elongated, and divided into basophilic granules (chromomeres) from which extend achromatic prolongations which become lost in the nuclear sap ("barbs" of the "brush" chromosomes: Duryee). The intimate structure of the brush chromosomes has been studied recently by Ris, who has shown that these chromosomes are in reality only a "misinterpretation of helically coiled structures."

After this brief resume of the morphology of the oöcyte in the growth phase, we can examine the studies devoted to its biochemistry: these researches are almost exclusively of a cytochemical order

and generally do not go beyond the descriptive and qualitative stage. We will consider later the few quantitative data at our disposal.

*a. Cytological Findings (Qualitative)*

Numerous investigators have tried to follow the synthesis of various reserve materials (yolk, fats, glycogen) in the oöcyte by utilizing cytochemical methods; let us remember that these techniques serve only to give us information on the localization of the substances investigated and can not furnish us with the facts for the chemical mechanism of their syntheses. In the case of the oöcyte, the cytochemical methods have contributed to the determination of the role of the various constituents of the cell (mitochondria, Golgi apparatus, nucleoli, etc.) in the formation of the yolk. The most important contributions in this field are those of Konopacki and Konopacka (frog, cuttlefish, ascidian, fish), V. and E. Marza (hen, fish), H. Hibbard (amphibia: *Discoglossus*), Jacquiart (spiders), etc.

Without analyzing these works in detail, let us try to find out the essential facts in the amphibians, which we shall take as the prototype: the young oöcytes are free of lipids, which do not appear as droplets until the cell is about 50  $\mu$  in diameter. They are first found at the margin of the "yolk nucleus," a cytoplasmic formation which appears to be chiefly mitochondrial in nature and which also takes part in the synthesis of yolk. The chemical constitution of the yolk nucleus has been studied especially by Voss, who showed (1924) that it exhibits selectively the indophenoloxidase reaction; therefore, this region contains oxidizing enzymes. Later (1927, 1931), Voss showed that the yolk nucleus is the only region of the young oöcyte that gives the "plasmal" reaction characteristic of certain phosphatides (acetylphosphatides of Feulgen and Bersin) (Fig. 9, left); the general reactions of fats are also positive in the region of the yolk nucleus according to Konopacki and Hibbard. It is interesting to note that some histologists (P. Gérard, Verne) suggest a relation between plasmalogen and certain oxidations: for Verne, for example, the plasmal reaction appears when one oxidizes certain lipid inclusions in a controlled fashion. Gérard has noticed that there is a frequent parallel between plasmalogen and lipid capable of transforming the Nadi reagent into indophenol blue. One can ask under these conditions whether the oxidases described by Voss are not simply lipids

of a particular type, with indophenol blue dissolving in the fatty inclusions; this reservation is the more justified since the further behavior of this "indophenoloxidase" is identical with that of lipids which form a ring around the periphery of the oöcyte then disperse in the cytoplasm between the vitelline particles (Fig. 9).

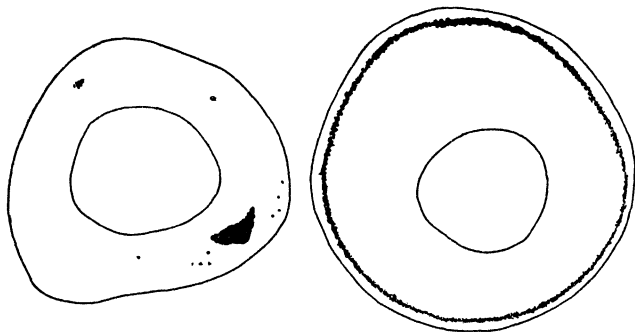


Fig. 9. Young oöcyte (*left*) and somewhat older oöcyte (*right*) of axolotl: plasmal reaction (Voss).

The origin of the protein yolk remains debatable: although Konopacki sees it originating in the mitochondria, H. Hibbard thinks that it precipitates inside of the vacuoles which stain with neutral red (vacuome of Parat). The question has led to a number of investigations, often contradictory, and it does not seem to be solved at present; it is regrettable, since any contribution to the mechanism of the synthesis of proteins, one of the big problems in biochemistry, would be very welcome. In any case, this synthesis occurs in the same places as that of the fats: it begins in the region of the yolk nucleus which breaks up into a peripheral ring; the yolk becomes distributed throughout the oöcyte, the largest particles located at the vegetal pole. Figure 10 is a schematic representation of the progress of yolk formation.

*Glycogen* makes its appearance shortly after the first lipid droplets and before the yolk. It grows progressively in quantity, but one always notices a particularly rich zone around the nuclei (ring of perinuclear glycogen). As to the germinal vesicle itself, it contains neither glycogen nor lipids nor indophenoloxidase.

The oöcyte is surrounded by a layer of "follicular" cells interposed between it and the blood capillaries. Do they play a direct

secretory role or do they simply form a sort of filter? We can not clearly answer this question although certain observation of Kono-packi and of Voss speak in favor of the first interpretation.

Brachet concerned himself with the localization of the sulfhydryl proteins during oögenesis in the amphibians; if one carries out the nitroprusside reaction after fixation with trichloroacetic acid, which coagulates the proteins as well as eliminating most of the glutathione, the following results are obtained: the young oöcytes give an intense reaction in both cytoplasm and nucleus. The yolk nucleus, when it appears, seems more highly colored than the remainder of the cyto-

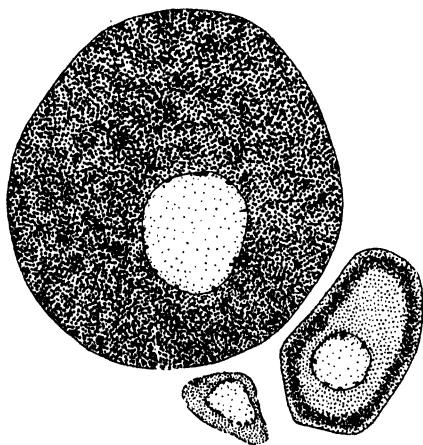


Fig. 10. Three stages in the origin of yolk in amphibia.

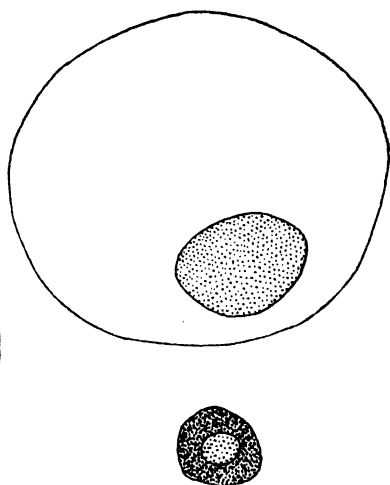


Fig. 11. Distribution of the sulfhydryl proteins at the beginning and at the end of oögenesis.

plasm. During rapid growth the reaction diminishes, especially in the vegetative half, rich in yolk; finally the colors are no longer obtained except at the animal cap. This condition shows that the yolk does not generally contain —SH groups in the amphibians although it has sulfur in noticeable quantities (Faure-Fremiet and Dragoiu). The nitroprusside reaction thus gives the inverse results of those furnished by the methods which reveal the yolk: the formation of the latter entails a continuous decrease in the areas containing sulfhydryl proteins and thus it seems that the localization of these coincides with that of the hyaloplasm. If one cuts these large oöcytes in half

and then tries the nitroprusside test, one finds that the nuclei give an intense reaction; the germinal vesicle, isolated by means of fine forceps, shows itself to be very rich in sulfhydryl proteins, but it is not possible to decide if the nucleoli take part in the reaction. According to Gersch, the amount of —SH groups in the nuclear sap increases during oögenesis and the nucleoli lose their sulfhydryl proteins. Brachet has been able to confirm these observations of Gersch on oöcytes sectioned in paraffin and stained by the new method of Chevremont and Frederic (reduction of ferric ferricyanide by the —SH groups); in *Triton cristatus* the staining of the cytoplasm is reduced during oögenesis although that of the nuclear sap is raised somewhat; the nucleoli are stained less deeply than the latter (Fig. 11).

The abundance of the sulfhydryl proteins in the germinal vesicle is certainly a very general fact: Brachet has found it in the pholads and the asteroids and it was found earlier by Dulzetto in the sea urchin; Ries has noted the same phenomenon in various other marine invertebrates, while Raven found it in the *Limnea*. Furthermore, tyrosine, like cysteine, tends to accumulate in the germinal vesicle of the oöcyte of the frog and particularly in the nucleoli (Serra).

The results relative to forms other than the amphibians will not concern us at length, since they do not differ much from those which have just been considered. Let us mention one interesting study of Jacquiart on the isolated yolk nucleus of spider oöcytes: its lamellar structure is revealed by polarized light; it is soluble in acids and in alkalis, it gives numerous protein reactions, in particular those typical of tyrosine and cysteine (—SH); it also contains a lipid, probably associated with proteins as a "complex"; the nature of this lipid is uncertain, but without doubt it behaves as a phospholipid. This author thinks that the yolk nucleus, which is rich in mitochondria, is a "site of intense secretion and the center of activity of the oöcyte." Tests were made by Marza and co-workers to determine quantitatively the amount of substances in certain structures by cytochemical means; the colorimetric method used permits only a gross approximation, and Caspersson's technique would undoubtedly furnish more precise values; the experiments of Marza are none the less valuable and have already led to some useful results. We point out, for example, that contrary to Macallum's opinion the germinal vesicle does not contain iron, a result since confirmed in the frog by Gersch; this metal plays a major role in cellular oxidations as we well know.

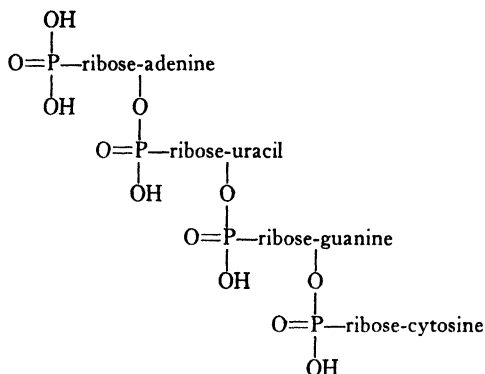
The yolk nucleus in the hen is poor in ash and does not appear to contain iron; this result does not agree with the role which we would have thought the yolk nucleus played in oxidations according to the observations of Voss on the localization of indophenoloxidase in the frog. The yolk nucleus contains plasmalogen in the hen as well as in axolotl.

If we try to summarize the facts acquired on the chemistry of yolk formation, we come to some very logical conclusions: the oöcyte is the site of an intense synthesis of reserve materials, such as proteins, fats, and glycogen. This synthesis occurs at the expense of the constituents in the blood through the intermediary of the follicular cells. It is difficult to ascertain the origin of these reserve substances, which appear suddenly in the oöcyte; the yolk nucleus, rich in mitochondria, doubtlessly is significant at the beginning but its real importance has not yet been appreciated. Unfortunately, the cytochemical studies can teach us nothing of the chemical mechanism of these syntheses, they show us only the topographical localization of the substances determined; it seems that the usual cytochemical techniques have already given all that one can attain, although surprises can always arise if we are able to determine new chemical compounds. We believe, however, that the progress in this field will result in the future through the growing application of quantitative methods.

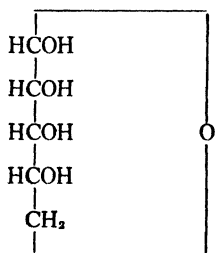
Before considering the results which such methods have already given us, let us pass to a review of what we know about the localization of *nucleic acids* in the oöcyte. Since we will have to make frequent allusion to these substances in following chapters, we believe it valuable to recall in a summary fashion their chief properties.

It is known that two principal nucleic acids exist: *thymonucleic acid* (desoxyribonucleic) and *ribonucleic acid*, the prototype of which is zymonucleic acid of yeast. On hydrolysis they give four molecules of  $H_3PO_4$ , four molecules of a sugar, two purine bases, and two pyrimidine bases. This combination forms a tetranucleotide, each mononucleotide resulting from the union of a molecule of  $H_3PO_4$ , a sugar molecule, and a molecule of a nitrogenous base. Here is the formula which Levene attributes to a tetranucleotide of yeast (zymonucleic acid); other formulas, perhaps more preferable, have been suggested by other authors and we refer for more detail to recent reviews by F. G. Fischer and by Gulland.

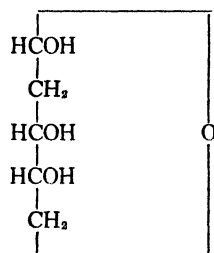
The carbohydrate in the two nucleic acids is not the same: in



ribonucleic acid it is a D-ribose, while in thymonucleic acid it is D-2-desoxyribose; as indicated by the formulae these two sugars differ only by one atom of oxygen; nevertheless the properties of ribose and desoxyribose permit their distinction from one another: the first alone yields furfural by hydrolysis which makes possible its microdetermination; the second recolors fuchsin decolorized by  $\text{SO}_2$  (to the exclusion of ribose) and this is the basis of the Feulgen reaction.



D-ribose



D-2-desoxyribose

Furthermore the two nucleic acids are different by the fact that uracil is replaced by its methyl derivative, thymine, in thymonucleic acid. It is necessary to emphasize the fact that, contrary to an all-too-general opinion, the two types of acid coexist in all cells, animal and vegetal; we will have occasion later to discuss their localization extensively. Recent work has established that the two types of nucleic acids can be distinguished by their physical properties: thymonucleic acid is found in its native state in the form of a high

polymer of tetranucleotides—its molecular weight is in fact about 500,000 to one million! (Signer and co-workers). Its molecule has an elongate form and therefore exhibits a birefringence of flow. It readily forms fibers which can be analyzed by X-rays (Astbury and Bell); it is probable that a number of nucleotides stack up to give a molecule of comblike form; it is curious that the distance separating the various nucleotides is the same as that separating the successive lateral chains in a completely extended polypeptide chain; as a result the nucleic acid combines easily with such a chain; one can picture with Astbury and Bell that the interval separating two nucleotides will exercise an effect on the structure of the protein with which they combine—in fact it has been observed that if thymonucleic acid is combined with a globular protein the latter opens up and is transformed into a fibrous protein. The molecular weight of the ribonucleic acid is certainly not as high, although it also forms a polymer of tetranucleotides: the recent work of Chantrenne on the ribonucleic acid of yeast, of Grégoire on that of the larva of the fly *Calliphora*, and of Cohen and Stanley on that of the tobacco mosaic virus all agree in showing that we are dealing with a compound of a molecular weight exceeding 10,000 and probably attaining 300,000. It now appears probable that ribonucleic acid in the native state possesses elongate molecules. Unfortunately the extreme lability of this compound in alkaline solutions makes it very difficult to study its physical properties. Gulland has proposed the following hypothesis to account for the differences in the properties of the two nucleic acids: while the tetranucleotides are placed end to end in rows in thymonucleic acid, they form a branching chain in ribonucleic acid. The structure of thymonucleic acid thus resembles that of cellulose, while that of ribonucleic acid is more like starch.

The nucleic acids in the cell are combined with basic proteins (protamines, histones) rich in diamino acids (arginine, histidine, lysine). The bonds uniting the proteins with the nucleic acids are still very poorly known; the fact that one can not separate the histone from thymonucleic acid by subjecting the thymonucleohistone to electrophoresis seems to indicate clearly that it is not a question of a salt linkage of the usual type (Carter and Hall, Mirsky and Pollister, Jeener).

In the course of the last few years the properties of thymonucleohistone extracted from the nuclei of various cell types has



been intensively studied (Carter and Hall, Mirsky and Pollister, Claude and Potter, Jeener, K. G. Stern, Pollister and Mirsky, von Euler and co-workers): this substance is formed of large elongate molecules, soluble in concentrated salt solutions and precipitated by distilled water. The problem of the chemical composition of the thymonucleohistone is complex and difficult; the use of varied physical methods by these investigators (dialysis, electrophoresis, ultracentrifugation, selective adsorption) shows that thymonucleic acid and the histone are joined to one another by labile bonds; it seems that the histone in its native state is a macromolecule, but it becomes fragmented rapidly into chains of very unequal length during the isolation of the nucleoprotein. From the chemical point of view the histone shows the peculiarity of containing little tryptophan; since nuclei from which the thymonucleohistone has been extracted still show a positive reaction for tryptophan, we must conclude that the nuclei contain proteins other than histones (chromosomin of Stedman). According to Mirsky and Pollister, the substance of the chromatin which they call chromosin, is made up of thymonucleic acid, histone, and proteins rich in tryptophan; these latter are generally of little quantitative importance, but nevertheless play an important role in the structure of the chromosome; in fact the chromosome retains its characteristic structure after the thymonucleohistone is quantitatively removed by a concentrated salt solution.

The addition of traces of calcium ions to thymonucleohistone fibers stimulates their contraction; Jeener succeeded by this means in simulating *in vitro* the changes undergone by chromosomes during mitosis. It is very interesting to note in this regard that the nuclei are much richer in calcium than is the cytoplasm (Williamson and Gulick, Scott).

Finally, let us recall that certain derivatives of ribonucleic acid are essential in the cellular oxidations and glycolysis, by functioning as codehydrases and cophosphorylases; these are both the mono- and dinucleotides with the base adenine (adenylic acid from muscle, adenosine triphosphate, coenzymes I, II, III).

Let us pass now to some studies on the *distribution of the two nucleic acids* during oögenesis. The question has been examined by a number of investigators in regard to *thymonucleic acid*. Here is a list, probably incomplete, of the authors who have applied the

Feulgen reaction to oöcytes: Koch (Chilopods), Voss (frog, lamprey, cat), Ludford (Limnea, rat, mouse), H. Hibbard (Discoglossus), J. Brachet (snail, clam, pholad, sea urchin, ascidian, triton, salamander, frog, lizard, guinea pig, sheep), Mukerji (insects), Gresson (insects), Bauer (insects, Echinoderms), Harvey (crab), Faulkner (Hydroids), Gardiner (*Limulus*), V. and E. Marza (hen, *Fundulus*), Konopacki (Ascidians), Gothié and Tsatsaris (mouse), N. K. Koltzoff (triton, hen, pigeon), Gersch (frog). The results obtained agree in major aspects; the Feulgen reaction is given exclusively by the *chromosomes*, which thus alone contain thymonucleic acid. The positive results sometimes seen in the cytoplasm (yolk of the amphibian: H. Hibbard, J. Brachet; hen: V. and E. Marza) are certainly not due to thymonucleic acid for they exhibit some abnormal characteristics. In the amphibians the color appears even without hydrolysis while in the hen the reaction no longer takes place after hydrolysis. The nature of the chemical constituent which causes this coloration of the yolk is not known; one suspects mainly the plasmalogen, the acetaldehyde which contaminates the alcohol used for histological technique (J. Brachet) or the potassium which shows the same topographical localization (Marza). One can conclude with certainty that the cytoplasm and the yolk of oöcytes are free of thymonucleic acid, like the cytoplasm of all normal cells.

Feulgen's reaction is also negative in the nuclear sap and in the nucleoli; however, Bauer has observed a slight coloration of the nucleoli in *Anopheles* and *Stegomyia* and Brachet has observed that in the frog the nucleoli become weakly Feulgen-positive at the time when they begin to break up during maturation. But these are exceptions and we must state as a general rule that the nucleoli of oöcytes are free of thymonucleic acid. It is important to note, however, that the nucleoli, especially at the time of their formation, are surrounded by a chromosomal ring staining intensely with Feulgen reagent (Fig. 12); this fact is observed very easily in the young amphibian oöcytes and is found again in the blastula at the time when the nucleoli reappear. The close attachments between the chromosomes and the nucleoli, which are known in a number of examples in cytology, argue in favor of the chromosomal origin of the nucleoli, a theory defended by McClintock and by Heitz.

The behavior of the *chromosomes* in regard to Feulgen's reaction is more important. Let us recall once more that the chromosomes

undergo a very particular change during oögenesis. Compact and staining deeply at first they soon elongate and thin out; often they fragment into a mass of granules arranged linearly (chromomeres), from which achromatic filaments extend into the nuclear sap (brush chromosomes). A little before maturation they again become more apparent, retract and regain their staining quality. During these morphological changes, the staining by Feulgen is also modified:

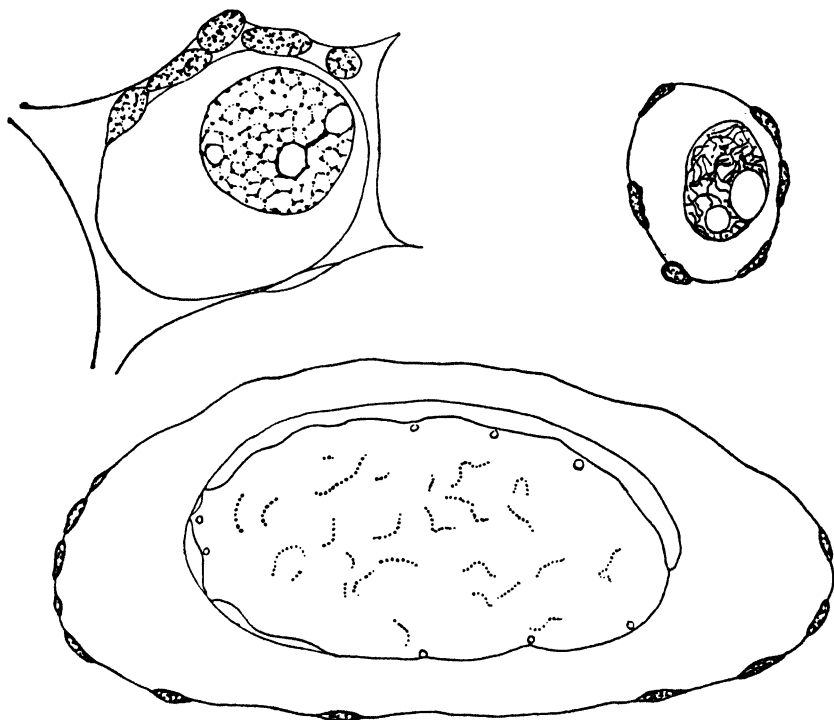


Fig. 12. Feulgen reaction during oögenesis in amphibia (H. Hibbard, J. Brachet).

the young oöcytes have a highly stainable nucleus, where the chromosomes appear deeply stained violet; but during the course of the rapid growth when the "brush" effect develops the Feulgen reaction tends to become negative; most of the authors think that the germinal vesicle is then completely free of thymonucleic acid. The latter does not reappear until the time when the preparation for mitosis of maturation begins.

The disappearance of the Feulgen reaction during the growth phase has been attributed either to a chemical transformation of nucleic acid present in the chromosomes or to an excessive dispersion of the chromomeres in the nuclear area; the particles charged with thymonucleic acid become too small to enable recognition of the violet stain. Brachet's observations on Urodeles led him to defend the first of these hypotheses (1929); the chromomeres are in fact, in these oöcytes, large enough so that one can find them easily even though they are not stained; but these chromomeres are not stained with Feulgen although iron haematoxylin shows them up very well. But Bauer (1933) showed that one can detect thymonucleic acid during oögenesis in insects if one takes care to use fixatives that prevent the swelling of the chromomeres and their partial dissolution during the hydrolysis. Brachet has been able to confirm these observations in the sipunculids and the phascolosomids (1937) and this led him to take up again the study of triton oöcytes. If the material is fixed in Zenker's or Allen's fluids, a close examination shows that the *chromomeres give the Feulgen reaction throughout oögenesis*, even when their dispersion is maximum during the growth phase. In the frog, where the chromomeres are much smaller and poorer in thymonucleic acid, the reaction can not be followed at the time when the chromosomes elongate, as Gersch has confirmed; a difference so fundamental between frog and triton is very improbable and we believe that the negative results, in the first case, stem chiefly from the reduced size of the chromomeres. This conclusion is the more reasonable since the oöcytes of the toad react like those of triton: the Feulgen reaction remains positive during oögenesis in the region of the chromomeres (Painter and Taylor). A complete disappearance of thymonucleic acid during oögenesis is the more unreasonable since it is possible to obtain a positive reaction if one centrifuges the oöcytes at very high speeds; the chromosomes gather together then at the centrifugal pole of the nucleus forming a mass which clearly stains with Feulgen's reagents. The question of whether the amount of thymonucleic acid of the nucleus shows quantitative variations during oögenesis remains open; what is well established, is that the thymonucleic acid is a constant constituent of the chromomeres, even during the growth phase. Figure 12 shows the condition of the oöcytes of triton stained with Feulgen.

We have discussed the question of the persistence of thymo-

nucleic acid in the chromosomes at some length because it has great importance from the point of view of the *chemical nature of the genes*. One generally tends to think that the latter are nucleoproteins and to attribute a role to thymonucleic during their reduplication in the course of cell division (see especially Timofeeff-Ressovsky, Friedrich-Frekxa); we will return later to this important question, making only the observation that this hypothesis is acceptable only if thymonucleic acid is a constant constituent of the chromosomes. It was for this end of resolving the problem of the relation between thymonucleic acid and the genes that the Russian geneticist Koltzoff took up in 1938 the study of oögenesis in triton, the hen, and the pigeon by means of the Feulgen reaction; he thinks that the chromosome is formed of a stable genotype, the "genonema," and of a phenotype susceptible to variation during development. The genonema constitutes a filament presenting a series of enlargements, the chromomeres; these only carry the genes and show great stability, morphological as well as chemical. Only agents causing mutation are able to alter them. Since, according to Koltzoff, thymonucleic acid disappears during the rapid growth of the oöcyte, it can not be a part of the genonema; it plays only a protective role and enters into the phenotypic structure, the "chromoplasm". This latter is impregnated with nucleic acid at certain times and thus it becomes recognizable under the microscope. The thickness of this protective envelope is very variable from time to time; the chromoplasm serves to ensure metabolic changes.

The concept of Koltzoff appears unacceptable: on the same material which he used (triton), Brachet has on the contrary detected thymonucleic acid in the chromomeres throughout oögenesis; the observations have been repeated and made under conditions which exclude all technical errors. From this one arrives at the contrary conclusion that thymonucleic acid is a *constant constituent of the chromomeres*, which correspond probably to the genes; if the distinction proposed by Koltzoff is justifiable, then we must consider the thymonucleic acid as one of the components of the stable element, of the genonema. The brush chromosomes of the oöcytes behave like the giant chromosomes of the salivary glands of *Chironomus* where bands rich in thymonucleic acid alternate with zones which do not contain it; and we admit in general that the bands stained by Feulgen correspond to the genes.

Let us now examine the *localization of ribonucleic acid during oögenesis*: Brachet remarked (1933) that the synthesis of nucleic acids in the sea urchin is not intelligible unless we admit the presence of a pentose-nucleic acid in the cytoplasm; we will see later the reasons impelling this conclusion. At this time, it was not possible to detect these substances in histological sections; the use of a ribonuclease (see Chapter I) now permits us to demonstrate conclusively that the cytoplasm of the oöcyte contains ribonucleic acid. If one treats the ovary of an amphibian with Unna's mixture (methyl green—pyronine), he finds that the pyronine stains the cytoplasm of the young oöcyte red; when yolk formation begins, the masses of yolk granules appear as unstained bodies in this red background. As the yolk invades the egg, the staining of the cytoplasm by pyronine decreases; it persists, however, as a well-colored perinuclear ring. At the end of oögenesis, the pyronine stains only this ring, a few granules scattered in the animal half of the egg, and the cortex. The total quantity of basophilic substance nevertheless remains significant, for centrifuging the egg (5–10 minutes at 3000 R.P.M.) condenses it between the fatty cap and the yolk mass. Furthermore, during all of oögenesis the nucleoli take up pyronine intensely. A treatment of the preparations with *ribonuclease* suppresses completely the basophily of the cytoplasm and the nucleoli without destroying these structures which still stain with eosin. The loss in basophily is thus due to the selective disappearance of ribonucleic acid: this acid is consequently localized in the cytoplasm (where its quantity decreases with development) and in the nucleoli of oöcytes. We shall see that the distribution of cytoplasmic ribonucleic acid is the same as that of sulfhydryl proteins; however, the nuclear sap is also rich in —SH groups although it contains no nucleic acid. These cytochemical findings have been confirmed by determination of the amounts of pentose in oöcytes: while the young oöcytes give on the average 0.206 mg % of furfural, the large oöcytes show only 0.065 and the isolated germinal vesicle 0.049. Figure 13 will give some idea of the distribution of ribonucleic acid during oögenesis.

Similar observations have been made in the fish (*Fundulus*) by Marza, Marza and Guthrie; the affinity of ribonucleic acid for pyronine not yet being understood, they did not give a chemical interpretation to their studies.

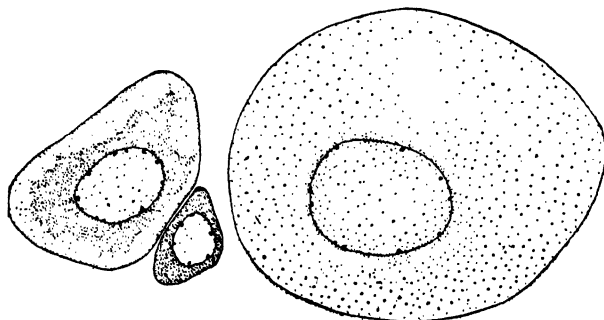


Fig. 13. Localization of ribonucleic acid in three stages of oögenesis in amphibia.

In addition, Brachet has found confirming evidence in mollusca (anodonts), in worms (planaria), in insects (hydrophilids), in mammals (rabbit), while Painter and Taylor have confirmed the results on the toad: the wealth of ribonucleic acid in young oöcytes, the progressive decrease of this substance with time are thus general rules; the presence of this acid in the nucleoli in variable quantities is also a constant feature.

Finally, these conclusions are in agreement with those of Schultz and Caspersson who carried out their studies on the absorption of ultraviolet light by the oöcytes of *Drosophila*; the nucleoli and cytoplasm give absorption spectra characteristic of nucleic acid and its concentration decreases in going from the nuclear membrane to the periphery; since it does not give the Feulgen reaction it must

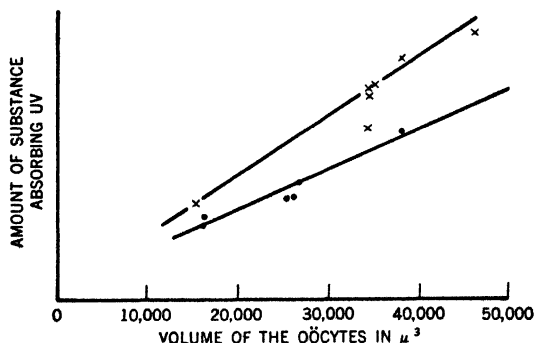


Fig. 14. Amount of ribonucleic acid in the cytoplasm of oöcytes of *Drosophila*: XX females (●) and XXY females (X) (Caspersson and Schultz).

be ribonucleic acid. The research of Caspersson and Schultz has established an extremely interesting point. *The amount of ribonucleic acid in the nucleoli and in the cytoplasm depends on the amount of thymonucleic acid and the chromosomal composition of the nuclei.* If one compares the oöcytes of females having a chromosome content

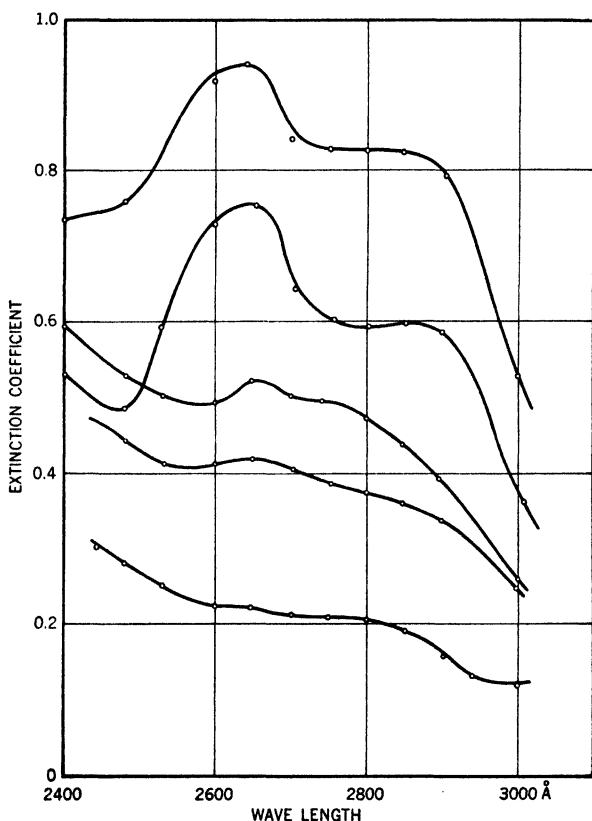


Fig. 15. Absorption spectra of nucleoli (upper two curves) and of nuclear sap in different regions of the nucleus (oöcyte of *Arenicola*). The band at 2650 Å is due to the presence of ribonucleic acid (Caspersson).

of XX and XXY one finds that the introduction of the heterochromosome Y increases the amount of ribonucleic acid of the cytoplasm. These facts are illustrated by Figure 14.

According to Caspersson (1940) the absorption spectrum taken at different regions in the nuclear sap varies, which suggests a dis-



continuity in the structure of the latter (Fig. 15); however, these measurements were carried out on oöcytes fixed in Carnoy and it is possible that artefacts are present since this fixative causes considerable shrinkage.

Concerning the presence of ribonucleic acid in the nucleoli, it is noteworthy that Gardiner (in *Limulus*) and Gersch (in the frog) have observed that these organelles contain phosphorus, while they appear to be free of iron and potassium. Gersch, on the basis of the solubility of the nucleoli in various substances believes that they are phosphoproteins; he excludes nucleoproteins only because the nucleoli are Feulgen-negative and he considers as possible their ribonucleoprotein nature. The observations of Caspersson and Schultz as well as Brachet leave no further doubt on this subject.

In conclusion let us say a word about the localization in the oöcyte of the *basic proteins* (histones) generally associated with the nucleic acids; the spectrographic findings of Caspersson and Schultz, as well as the results from Serra (detection of arginine), agree in showing that the nucleolus of the oöcytes is rich in histones. We have found the same fact for the ovary of Batrachians: the arginine reaction shows a maximum intensity in the region of the nucleoli while the nuclear sap gives only a faint color. The cytoplasm of young oöcytes, rich in ribonucleic acid, shows an intense color by the method of Serra; however the reaction is also very strong in the region of the yolk granules, especially those at the vegetal pole. There results, in the large oöcytes, an extremely sharp vitelline gradient.

#### *b. Histochemical Observations (Quantitative)*

The quantitative studies on the metabolism of the oöcyte during growth are, unfortunately, still small in number; concerning respiration, for example, we have only the work of Mestcherskaja on the oöcytes of fishes and amphibians. This author classifies oöcytes into three groups according to size: I, oöcytes from 15 to 225  $\mu$  in diameter, still transparent; II, oöcytes from 225 to 425  $\mu$  in diameter where formation of the yolk and fat begins; III, oöcytes of 425 to 900  $\mu$ , rich in yolk and pigment (the measurements were made chiefly on the ovary of the frog). In short it concerns oöcytes taken before yolk formation, at the beginning of this process and when it reaches its maximum.

If the oöcytes are stained with neutral red, one observes in going from stage I to II a change in the direction of alkalinity; however, we have already seen that the measurement of pH by vital staining is subject to a number of criticisms. If one treats the eggs with vital dyes capable of decoloration by reduction (oxidation-reduction potential indicators, like methylene blue, brilliant cresyl blue, Janus green) and places them in a current of hydrogen, they become decolorized at different rates; the decoloration takes place in 3-9 minutes for oöcytes of stage II, in 35-45 minutes for those of small size (stage I). Unfortunately the author chose a temperature of 32-34° C., which is higher than that in which frogs normally live. Nevertheless these experiments are purported to show that the respiratory activity is particularly high when yolk formation begins; this interpretation is confirmed by direct measurements of the oxygen consumption of isolated oöcytes carried out with the microrespirometer of Kalmus (an instrument comparable to that of Gerard and Hartline, described in Chapter I); the oöcytes at stage I absorb 0.69 mm<sup>3</sup> of oxygen per cubic millimeter of oöcyte, while the values for stages II and III are 1.5 and 1.2, respectively; thus at stage II, in the oöcyte with intense yolk formation, the respiration is greatest. The decrease which takes place later results no doubt from the huge accumulation of yolk, inert from the point of view of respiration. The author estimates that the mass of "active protoplasm decreases somewhat about half way through the course of the formation of the yolk. Finally, Mestcherskaja has stated that the sensitivity of the oöcytes to various agents changes during growth: the small ones are affected to a greater degree by cyanide, HgCl<sub>2</sub>, and hypotonicity; the medium size oöcytes are less resistant to heat while the large ones are particularly sensitive to alcohol.

These investigations, in spite of their incompleteness, are valuable because they indicate a particularly intense respiratory activity at the time of most active protein synthesis; it is very desirable that these measurements be repeated and completed by measurements of the respiratory quotient, of glycolysis, etc.

A few studies have been devoted to the *relative role of the nucleus and the cytoplasm in the respiratory exchange* of the oöcyte.

The opinion holding that the nucleus is the center of cellular oxidations has been expressed frequently; it was developed in the first place by Jacques Loeb, when he noticed that enucleated Protozoa

did not regenerate. He attributed this to the fact that a lowering of the oxidations below a limiting value took place, but he had no proof of this fact. Loeb's concept has received support from observations by R. S. Lillie, who, using cytochemical methods, noted that the indophenoloxidase is frequently localized in the nucleus or very close to it. As a matter of fact Lillie's conclusions have not been confirmed by other investigators: according to Graff, the enzyme is never found in the nucleus, although it may be localized close to the nuclear membrane. Katsunuma, who studied a number of organs, found indophenol oxidase in the nucleus in only a single case, that of the Purkinje cells in the cerebellum. Also, Van Herwerden never found indophenoloxidase in the nuclei of sea urchin eggs (although the cytoplasm contains it) and has observed that the enucleated fragments of holothuroidian eggs still give the reaction 18 hours after the operation. The peroxidase activity of the nucleus, one time claimed, seems to be an injury phenomenon (M. Prenant, J. Brachet 1934, Lindahl).

The hypothesis of the *nucleus as a center of oxidations* would no longer merit attention if it were not supported by some celebrated experiments of Warburg. In 1909 this author showed that the nucleated red blood cells of the bird respired much more rapidly than the enucleated corpuscles of man or rabbit. If one centrifuges the red blood cells of birds after hemolysis, the residue alone absorbs  $O_2$ —and it contains the nuclei with other cellular debris; homogenizing this residue causes a rapid disappearance of respiratory activity. These experiments do not appear to be as convincing as one might believe. In the first place there is a comparison of the cells of different species; besides the enucleated blood cell is destined for a rapid death; finally Warburg himself has shown that immature red blood cells of mammals (reticulocytes) respire much more than mature ones; the reticulocytes, however, are enucleated and their increased respiration appears to relate to their content of basophilic substances (of ribonucleoproteins, in fact, as has been shown by Caspersson and P. Dustin). Finally, Warburg showed in 1910 that the increase in oxygen consumption which occurs during segmentation is not proportional to the increase in number of nuclei; we will see later that this opinion has been adequately confirmed. This is why Warburg (1914) came to the conclusion that the respiration of the sea urchin egg depends on the cytoplasm and not on

the nucleus; it bears a relation chiefly to the quantity of "structures" in the egg, which goes hand in hand with basophily, and here again the basophilic material doubtlessly corresponds to the ribonucleoproteins. One sees that under these conditions the question of the role of the nucleus in cellular oxidation remains entirely open.

A first trial to attack it experimentally was made by Rapkine and Wurmser. Measuring the oxidation-reduction potential ( $rH_2$ ) in anaerobiosis, they observed no differences between the nucleus and the cytoplasm in the sea urchin oöcyte and in the salivary gland cells of *Chironomus*. The authors concluded that the rate of activation of oxygen is no more intense in the nucleus than the cytoplasm but one can not thereby exclude a difference in the respiration

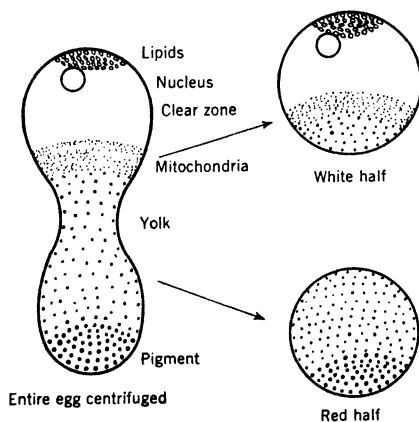


Fig. 16. Ultracentrifugation of unfertilized egg of *Arbacia* (E. B. Harvey).

of the two; Rapkine and Wurmser, however, considered unlikely the possibility of oxidations in the nucleus at the expense of free oxygen. The question has been taken up by Chambers and co-workers, who arrived at some different results; the nucleus is completely incapable of reducing redox dyes or oxidizing the reduced form of injected dyes: it is thus absolutely inert from the point of view of oxidation-reduction and the authors naturally side with the opponents of Loeb's theory. It is evident that the methods used (injection of dyes) can not give us information on the rate of metabolism of the nucleus and the cytoplasm and that a more direct study is still desirable.

The first trial made from this viewpoint is that of Shapiro

(1935), who has compared the oxygen consumption of nucleated and enucleated fragments of sea urchin eggs; the separation of the two fragments has frequently been made following the method of Harvey, which consists in centrifuging the eggs at very high speed in a solution of the same specific gravity (isopycnotic). The centrifugation causes a stratification of the contents of the egg which then elongates, taking a dumbbell shape and finally separating into two fragments (Fig. 16); the "light" or "white" half contains the fats, the nucleus, and the transparent cytoplasm rich in ribonucleic acid (Harvey and Lavin); the "heavy" or "red" half contains the remainder of the hyaline protoplasm, the yolk, and the pigment. This latter is echinochrome, a red pigment present in the eggs of certain sea urchins, notably *Arbacia*. This heavy fragment is thus opaque, pigmented, and always free of a nucleus.

One would expect to find a higher metabolism in the light fragments, containing the nucleus and a large part of the "active" cytoplasm, than in the heavy fragments, filled with yolk and pigment; but Shapiro found exactly the opposite (Fig. 17): oxygen consump-

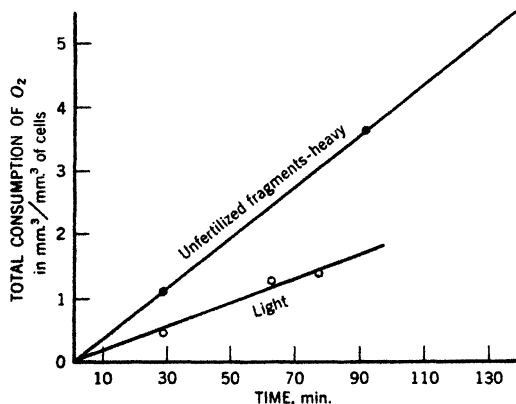
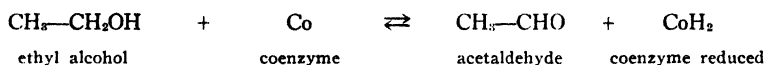


Fig. 17. Oxygen consumption of unfertilized "light" and "heavy" fragments in *Arbacia* (Shapiro).

tion (expressed in cubic millimeters per 10 mm<sup>3</sup> of eggs per hour) is on the average  $1.23 \pm 0.07$  for the light half and  $2.81 \pm 0.15$  for the heavy fragment. The respiration of the nucleated light fragment is of the same order of magnitude as for the entire egg, while that of the enucleated heavy portion is greater by 88%. These inter-

esting experiments show that the centrifugable granules play an important role in the oxidations of the egg; they establish further that the nucleus can not have a preponderant importance from this point of view.

The work of Shapiro has been extended by some observations on the distribution of various respiratory enzymes in the eggs cut in two by Harvey's method; before examining them, let us summarily recall the way in which oxidations take place within the living cell. The oxidizable substances (substrates) of the cell are first subjected to a dehydrogenation by the action of *dehydrogenases*. These enzymes are proteins which function only in the presence of a coenzyme; the latter are dinucleotides formed of phosphoric acid, ribose, adenine, and another nitrogenous base which plays an important role in the dehydrogenation: the amide of nicotinic acid (coenzymes I and II) and isoalloxazine (coenzyme III). The hydrogen of the substrate passes to the coenzyme which is thus reduced, while the substrate is oxidized at the same time; for example, the oxidation of alcohol to acetaldehyde goes this way:



The reduced coenzyme then is oxidized in transferring its hydrogen to a chain of hydrogen transporters (*carriers*) which are alternately reduced and reoxidized; thus the hydrogen is passed to the coenzyme of diaphorase (a protein whose active group is coenzyme III: isoalloxazine), then to succinic dehydrogenase (which catalyzes the transformation of succinic acid to fumaric acid), to the cytochromes (red pigments with an active group of an iron haemin base), and finally to cytochrome oxidase (probably identical to the indophenoloxidase of the cytochemists) which assures the combination of the hydrogen and oxygen to form water.

Let us return now to the oxidative enzymes (*desmolases*) of centrifuged sea urchin eggs. Navez and Harvey have examined the distribution of indophenoloxidase in the two fragments by following the oxidation of *Nadi* colorimetrically (see Chapter I): they state that conforming to the findings of Shapiro, the heavy fragment, enucleated and rich in granules, contains more than the light half. Navez has also noticed that the eggs contain a lipid which oxidizes *Nadi* and which accumulates in the light half; the fact shows once

again how dangerous this method is for the detection of oxidases. Later, Ballentine studied the distribution of *dehydrogenases* in the two fragments. He also ascertained a heterogenous distribution, the heavy fragment containing 55% more dehydrogenases than the other; since this heavy portion represents only 45% of the entire volume of the egg, he concluded that the enucleated part possessed about 70% more dehydrogenase activity than the nucleated fragment at equal volume. A high proportion of desmolases are thus bound to the granules displaced by rapid centrifugation and it is evident that the nucleus can contain only small amounts.

The idea that cytochrome oxidase accumulates in the heavy fragment and that it is bound to cytoplasmic granules, receives support from some recent work of Shapiro (1940), who studied the cyanide sensitivity of the two types of fragments; it is known that this poison combines specifically with cytochrome oxidase: and the respiration of the heavy half is strongly inhibited by cyanide which has hardly any effect on the oxygen consumption of the light fragments. However the determinations of cytochrome oxidase carried out by Hutchens, Kopac, and Krahl on eggs homogenized and centrifuged at various speeds indicates that two-thirds of this enzyme is found in the hyaloplasm; the yolk contains only 12% and the mitochondria 10%.

This important American research constitutes a beautiful contribution to cellular physiology; it does not, however, resolve the question of the role of the nucleus in oxidations in as complete a manner as we hoped because the light fragment contains, in addition to the nucleus, the lipids and some hyaline cytoplasm; besides, one fears that the centrifugation may alter the relations between the various substances in the chain of oxidations and thus arrive at false results.

For this reason Brachet (1937, 1938, 1939) has attacked the problem by using other material, the oöcyte of the frog; it is really very easy to separate the nucleus (germinal vesicle) of these oöcytes by means of a fine forceps and to clean the isolated germinal vesicle from the adhering cytoplasm by a rapid washing. The research began with a study of the occurrence of respiratory enzymes in the nucleus by use of conventional cytochemical techniques; the indophenol oxidase reactions, as had been shown earlier by Voss, and the peroxidase reaction were regularly negative; these observations were

verified later by Gersch. Since according to this author the germinal vesicle does not contain iron, it seems clear that the amount of haemins in the nucleus must be very low. However, it was often found that the isolated nucleus between slide and coverslip is able to decolorize methylene blue; this fact had already been observed by Waterman in the case of the germinal vesicle of the oöcytes of *Sabellaria*.

These cytochemical observations suggest the idea that the nucleus is without doubt not free of all respiratory activity, but there is no indication that the latter can be considerable. This opinion is completely confirmed by *direct measurements of the respiration of isolated nuclei*. Oxygen consumption has been measured in *Triturus pyrrhogaster* with the aid of the Gerard-Hartline respirometer, determinations being made on 6–20 isolated nuclei; a microtitration or the colorimetric measurements of the pH permits us to follow CO<sub>2</sub> production. These quantitative experiments have shown that oxygen consumption is low, but that it is maintained constant for 5–6 hours; it is about 0.002 mm<sup>3</sup> per nucleus per hour, while that of the entire oöcyte is 0.17 mm<sup>3</sup> per hour; thus we see that the respiration of the nucleus represents only 1 to 1.5% of the gaseous exchange in the cell. Similar results are found when one measures the CO<sub>2</sub> production, which is about 0.0018 for the nucleus and 0.13 for the intact oöcyte per hour; further it has been ascertained that the enucleation of the oöcyte produces only a negligible decrease in the elimination of CO<sub>2</sub>: 0.12 mm<sup>3</sup> of CO<sub>2</sub> per hour in place of 0.13.

These results weaken all the claims that the germinal vesicle is the center of respiratory metabolism of the oöcyte; we have made sure that the weak respiration is not a matter of insufficient substrate; the addition of glucose, of cytoplasm taken from the egg, has no effect whatever on the metabolism. Let us add here that modifications in the composition of the saline medium in which the isolated nucleus is placed, notably the addition of calcium which strongly modifies the colloidal state (Duryee), does not influence the rate of oxidations.

It is evidently premature to hope to draw any general conclusions at this time. The results of the American authors and of Brachet complement each other very satisfactorily and one can certainly say that in the oöcytes and in the unfertilized eggs the nucleus assumes only a minor role in the oxidations. But it is important to note that



we are dealing with nuclei of a special type, poor in chromatin and with a large amount of nuclear sap; there is no proof that the same situation applies in the case of ordinary nuclei much richer in thymonucleic acid. The indications at our disposal in this regard are again contradictory: recent experiments of Dounce and Lan have established that the nuclei of red blood cells hemolyzed by saponin and washed retained a high oxygen consumption even in the absence of substrate; thus they should contain a relatively complete system of oxidation. But, according to Dounce, the nuclei of rat's liver are very deficient in succinic dehydrogenase, in cytochrome c, in coenzyme, in riboflavin in appreciable amounts, cytochrome oxidase and lactic dehydrogenase. Catalase appears also to be lacking in the nuclei of the salivary glands of *Chironomus* although it is found in abundance in the cytoplasm, particularly in the hyaloplasm (Bunding). The results of Dounce on the nuclei of liver have been extended by von Euler, Fischer, Hasselquist and Jaarma to isolated nuclei of thymus and of Jensen's sarcoma. The Swedish authors have found only very small amounts of lactic dehydrogenase, succinic dehydrogenase, catalase, and coenzyme. While Boell, Chambers, Glancy and Stern have ascertained an oxidative activity in the nuclei of salivary glands of *Chironomus*, Zittle and Zitin observed that the head of bull sperm is very poor in cytochrome oxidase and in iron compared with the flagella and especially the middle piece. Dounce found this enzyme in the nucleus of rat's liver, but in much smaller amounts than in the cytoplasm. In contrast to what this author has found in rat's liver, succinic dehydrogenase is found in large quantities in the nuclei of the liver of *Batrachians* according to Lazarow.

We see that new investigations are necessary before the situation becomes clarified; the actual discrepancies doubtlessly arise in great part from the fact that different authors have used different methods of isolating the nuclei: certain of these manipulations run the risk of altering the enzymes to be studied, while in other cases the separation of nuclei from cytoplasmic debris leaves much to be desired. It is certain that the coming years will see our knowledge of this interesting field of cellular physiology greatly enriched.

We have restricted ourselves so far to the studies on the respiratory metabolism of the nucleus and of the cytoplasm: what is known of the distribution of the *hydrolases*, that is, the enzymes catalyzing hydrolytic splitting? The question has been chiefly

studied by Holter and Linderstrøm-Lang in the case of invertebrate marine eggs and ameba; by Duspiva and by Brachet for the frog's oöcytes. In 1933 Linderstrøm-Lang focussed his attention on the distribution of dipeptidase in the egg of *Dendroaster*; this enzyme breaks the peptide linkage  $\text{—CO—NH—(R—CO—NH—R}_1\text{ + H}_2\text{O = RCO}_2\text{H + R}_1\text{NH}_2\text{)}$ , breaking alanyl-glycine into alanine and glycine. Linderstrøm-Lang cut the unfertilized eggs in two with a micromanipulator and ascertained that the two fragments, nucleated and enucleated, contain the same amount of enzyme. Linderstrøm-Lang and Holter as well as Holter have compared the dipeptidase activity of nucleated and enucleated fragments obtained by centrifugation of the eggs of different species (*Arbacia*, *Echinarachnius*, *Psammechinus*, *Chaetopterus*). Here again the enzyme is distributed in a homogenous fashion and the nucleated fragment has no more activity. One sees that although the distribution of the desmolases is changed by centrifugation, that of dipeptidase is not affected by this treatment; the latter enzyme is thus found in a diffuse state in the hyaline cytoplasm. One must also take note that the measurements by the Danish authors have been carried out on eggs which have completed maturation so that if the germinal vesicle were rich in dipeptidases they would spread into the cytoplasm with the nuclear sap during maturation.

These results obtained on unfertilized eggs have been confirmed in ameba (Linderstrøm-Lang and Holter); here again the dipeptidase appears to be distributed in a homogenous fashion in the hyaline protoplasm (Holter and Kopac); it is interesting to note that the enucleation of the ameba with a micromanipulator hardly decreases the amount of dipeptidase in the cytoplasm even 24 hours after the operation. One desmolase, catalase, which decomposes peroxide into oxygen and water, behaves like dipeptidase; it is found in no greater quantities in the nucleated fragment than in the cytoplasm free of nuclei. However, in the case of amylase, which converts starch into reducing sugars, there appears to be a difference: this enzyme accumulates after centrifugation in the regions where the mitochondria are most numerous and it is probable that it is localized in the mitochondria; its behavior during centrifugation differs completely from that of the dipeptidase (Holter and Doyle), but we find no indication in favor of a particularly rich enzyme content of the nucleus.

Summarizing, the important works of Linderstrøm-Lang, Holter,

and their collaborators demonstrate that the hydrolases *do not accumulate in the nuclei*; these enzymes can be found either in the diffuse state in the *hyaline cytoplasm* or in the *mitochondria* (amylase in ameba); it may even be that in the first case the enzymes are secreted by the mitochondria and diffuse into the neighboring cytoplasm. The localization of the hydrolases, with the exception of amylase, thus differs profoundly from that of the desmolases studied by the American investigators.

The study of the distribution of a few hydrolytic enzymes between the nucleus and the cytoplasm of the ultracentrifuged egg has been pursued by D. Mazia recently. The amount of acid phosphatase of the nucleated fragment is less than that of the enucleated half; on the contrary the nucleated fragment is by far the richer in thymonuclease, the enzyme which depolymerizes the large thymonucleic acid molecules. Since the amount of thymonuclease of the egg increases after fertilization, Mazia concluded that this enzyme was localized in the region of the chromosomes. However this conclusion appears to us to be hasty because there is no proof that the thymonuclease in the nucleated fragment is really in the nucleus and not in the hyaloplasm; this doubt is the more justified since Mazia can not demonstrate with certainty the presence of thymonuclease in spermatozoa, much richer in chromatin than the nucleated egg fragments. The experiments of Mazia indicate only that the cytoplasmic inclusions are free of this enzyme.

It is valuable to compare these conclusions with those drawn from direct studies of the amount of enzymes in the nucleus of the frog; we have seen that the case of the germinal vesicle has not been investigated by Linderstrøm-Lang and Holter; its interest rests in the fact that it was considered as a reserve of enzymes that become dispersed in the cytoplasm during maturation (Wilson, Mathews): this hypothesis rests only on cytological arguments, without any chemical basis. Concerning *dipeptidase*, Brachet (1938) has found that this enzyme is present in the germinal vesicle in easily measurable quantities; however, the cytoplasm of large oöcytes is somewhat richer in dipeptidase than the nucleus in spite of its high yolk content. Thus the germinal vesicle does not contain a reserve of dipeptidase. As to the young oöcytes in which yolk formation has just begun, they contain about three times more of the enzymes per unit weight as compared with the large oöcytes.

Before examining the behavior of other hydrolytic enzymes studied at this time, let us analyze the work which Duspiva has recently devoted to the dipeptidase of frog oöcytes: his experiments were carried out on *Rana esculenta* and *Rana fusca* (Brachet studied the second species) at different stages of oögenesis. Duspiva has improved the technique of removing the nuclear sap by pipetting the latter out of the nucleus preserved under a drop of oil; this procedure eliminates an error due to the loss of dipeptidase which comes out of the nucleus when it is washed in physiological fluid. If one measures the amount of enzyme present in the oöcyte at various stages of growth, one notices (Fig. 18) a sharp increase in

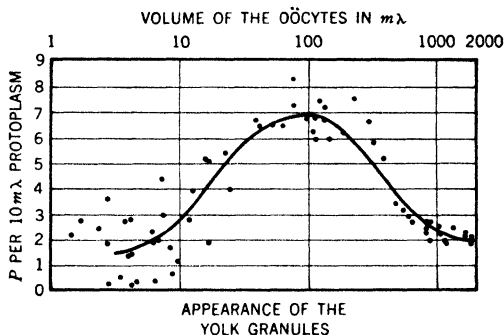


Fig. 18. Dipeptidase activity of cytoplasm during oögenesis in the frog (Duspiva).

the concentration in the *cytoplasm* at the time when yolk formation begins; this makes very probable the participation of dipeptidase in the synthesis of proteins, conforming to the opinion of Linderstrøm-Lang and Holter. The maximum amount is attained at the time when the yolk forms a ring at the periphery of the oöcyte; later a decrease in the concentration of the enzyme occurs by reason of the increase in the yolk (which is free of dipeptidase as affirmed by Duspiva). It is well to emphasize the parallelism between the observations of Duspiva and Mestcherskaja: the amount of dipeptidase attains a maximum in the stage of oögenesis exactly where the oxidations reach their height.

Regarding the amount of dipeptidase in the *nucleus*, Duspiva observed no direct relationship with yolk formation: in *Rana fusca* (Fig. 19), the nucleus contains only a small amount of the enzyme

the concentration remaining constant throughout oögenesis. In *Rana esculenta* (Fig. 20) the germinal vesicle is richer in dipeptidase, and in exceptional cases may contain more than the cytoplasm. However, the graph and figures of Duspiva show such variability in this species that it is difficult to draw rigid conclusions.

Let us mention some more results of Duspiva: the addition of various substances able to activate dipeptidase does not change the values obtained; thus there are no large amounts of an inactive form of the enzyme, either in the nucleus or the cytoplasm. One other fact, the oöcytes contain a *polypeptidase* (able to hydrolyze a tripeptide). Here again the nucleus contains less of the enzyme than the cytoplasm.

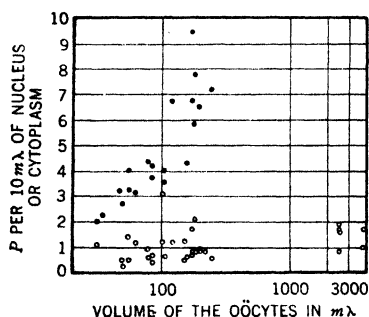


Fig. 19. Dipeptidase activity of nucleus (○) and cytoplasm (●) in *Rana fusca* (Duspiva).

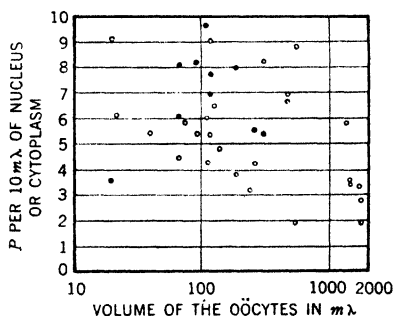


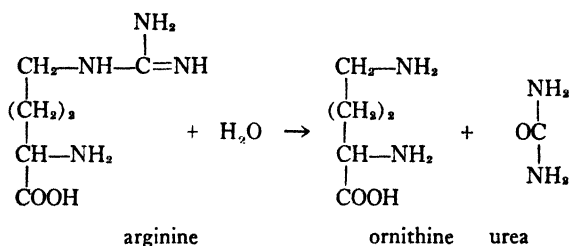
Fig. 20. Dipeptidase activity of nucleus (○) and cytoplasm (●) in *Rana esculenta* (Duspiva).

We see that our conclusions are not changed much by the investigations of Duspiva who used precise methods. Peptidase without doubt plays an essential role in the cytoplasmic formation of yolk, apparently without a direct participation of the nucleus in this phenomenon. The significance of this conclusion will be seen when we discuss the theories which have attributed an important activity of the nucleus in the synthesis of proteins (Caspersson).

Let us pass to an examination of the distribution of other hydrolases between nucleus and cytoplasm of the frog oöcyte; Brachet has found that esterase (which breaks ester linkages, *e.g.*, in the glycerides) is present in only small amounts in the oöcytes; the nucleus contains only a trace, while the cytoplasm contains about twice as much. The young oöcytes in which yolk formation has

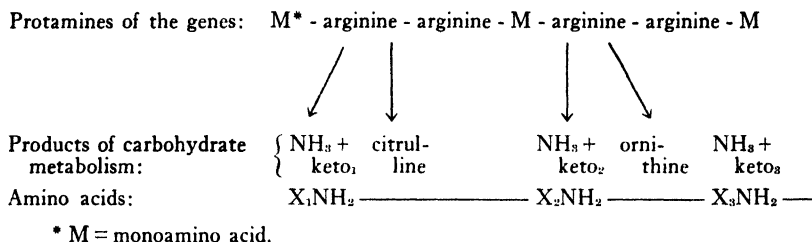
begin have a much higher esterase content. These observations, on the whole, are logical: the young oöcytes have been taken at the time when synthesis of fats attains its climax; as to the germinal vesicle, it appears free of lipids and it is not surprising that it contains hardly any esterase. Let us add that Behrens has not found lipase in nuclei extracted from liver and isolated by fractional centrifugation.

Brachet's studies have recently been extended (1942, 1944) to a series of enzymes able to take part in the hydrolysis and synthesis of nucleoproteins: These are *alkaline phosphatase*, *ribonuclease*, and *arginase*. The first hydrolyzes all the phosphate esters; however, it does not appear that it would be able to remove the phosphate from nucleic acid; the latter would first need to be depolymerized, then transformed into monucleotides by the nucleases (we know that ribonuclease belongs to this enzymatic system). As substrates, hexosediphosphate, thymo- and zymonucleic acids were used. As to arginase, it transforms arginine into ornithine and urea.



This enzyme is found chiefly in liver, where it plays an important role in ureopoesis (Krebs and Henseleit), but Edlbacher has reported that it is also found in actively growing tissues (tumors, embryos). However the more recent and more complete investigations of Greenstein and his co-workers have established that the amount of arginase does not necessarily increase during the course of the development of cancer: the generalization made by Edlbacher was thus premature and not exact. According to Edlbacher, arginase takes part in the synthesis of the protein portion of the nucleoproteins which is rich in arginine; this author even believes that arginase permits the synthesis of specific proteins, constituting the genes, by detaching from arginine one or two of its active  $\text{NH}_2$  groups and by transferring them to keto acids coming from carbohydrate

metabolism; thus they become transformed into amino acids, then into proteins. The scheme below represents Edlbacher's hypothesis:



Edlbacher visualizes the synthesis of proteins as an autocatalysis determined by the structure of the chromosomal proteins, in particular, by the position of the molecules of arginine; arginase would control this process to which we will return later on.

It was definitely indicated to Brachet to investigate to see if the germinal vesicle contained arginase, since it is a question of its occurrence in a cell which actively synthesizes proteins. In spite of the use of an extremely sensitive technique (determination of urea by means of urease in the Linderstrøm-Lang Cartesian diver), no arginase was found in the frogs oöcytes either in the young oöcytes where yolk formation was beginning or in the larger cells; nor was it possible to demonstrate the presence of arginase in the germinal vesicle even with activators. Thus the mechanism pictured by Edlbacher does not seem to have much of a role in the oöcytes of Batrachians. As for ribonuclease, the enzyme is found in detectable amounts and equally distributed in the nucleus and the cytoplasm of large oöcytes; the younger ones contain almost double the amount per unit volume. The germinal vesicle is not able to remove phosphate from nucleic acids although it easily attacks hexosediphosphate; it thus contains a phosphatase but some of the enzymes necessary for the complete hydrolysis of nucleic acid are doubtlessly lacking. The cytoplasm acts essentially the same as the germinal vesicle but it is further able to liberate phosphate from the yolk; the presence in the yolk granules of an enzyme liberating phosphate from phosphoproteins (phosphoproteinphosphatase) has also been described by Harris. As to the young oöcytes, they are richer in phosphatase than the larger ones and they are, moreover, able to completely hydrolyze zymonucleic acid to the exclusion of thymonucleic acid.

These observations show that the nucleus contains only a small quantity of the hydrolases present in the oöcyte; they demonstrate further that the yolk is an inert material, enzymically speaking, although it may serve as a substrate for phosphatases. During oögenesis, there appears to be a synthesis of hydrolases which is followed by an apparent diminution due to the fact that the egg becomes filled with inactive yolk.

Let us quickly examine how far these conclusions obtained in the case of the oöcyte are confirmed in the cases of ordinary cells: Dounce, studying rats' liver has found that the nuclei are less rich than the cytoplasm in arginase, in acid phosphatase and in esterase. However, they contain more alkaline phosphatase than the cytoplasm. The latter observation, established by chemical determinations, sees support by the cytochemical observations of Moog, of Krugelis, and of Willmer who have determined the phosphatases in the cells by the histochemical technique of Gomori: in various types of cells (chick embryo, spermatocytes) the phosphatase reaction is particularly intense in the region of the chromosomes. The difference between these nuclei and the germinal vesicle is probably due to the fact that the latter contains much less chromatin and much more nuclear sap. In fact, Brachet (unpublished observations) has noticed that only the nucleoli and the brush chromosomes give the cytochemical reaction of Gomori for alkaline phosphatase in the case of the oöcytes of *Batrachians*. The richness of alkaline phosphatase in chromosomes has recently been confirmed by Danielli and Catcheside: applying Gomori's method for the detection of this enzyme in the particular case of the giant salivary chromosomes of *Drosophila*, the English authors observed an accumulation of the enzyme in the bonds rich in thymonucleic acid. The amount of this acid and of the phosphatase of different bonds is not always absolutely parallel. The topographic coincidence between the points where the genes are located and those where the enzyme is active constitute for Danielli and Catcheside an indication of a process by which the genes influence cellular activity. Let us add that Krugelis has reported that the giant chromosomes of *Drosophila* are rich in alkaline phosphatase; but according to this author there is, on the contrary, a good parallel between the amount of nucleic acid in the chromosomes and their alkaline phosphatase activity. Note, in any case, that there does not appear to be a direct bond between the



enzyme and thymonucleic acid; in fact, one removes the thymonucleic acid from the nuclei by means of thymonucleodepolymerase without appreciably decreasing the intensity of the phosphatase activity (unpublished observations of Brachet); further, Jeener has shown that the alkaline phosphatase of nuclei is bound to the ultracentrifugable granules and these granules are relatively poor in thymonucleic acid.

Before leaving this question of nuclear localization of alkaline phosphatase, let us consider two recent observations which are of interest: first of all Krugelis has observed that chromatin, but not cytoplasm, is able to liberate inorganic phosphate when one uses partially depolymerized thymonucleic acid as substrate; on the contrary, ribonucleic acid constitutes a good substrate for the cytoplasm although it is not attacked by the nucleus. This curious result should undoubtedly be related to the fact that thymonucleic acid is found localized in the chromatin, while ribonucleic acid is a constituent of the cytoplasm and the nucleolus; however, the latter is able to attack thymonucleic acid.

In addition, Brachet and Jeener have shown that the intensity of the alkaline phosphatase reaction varies greatly in the nuclei of different tissues of the mouse. The splitting of glycerophosphate is rapid in the intestinal mucosa, slower in the testes, and very weak in the kidney, the brain and especially in muscle and the erythrocytes of birds. And there exists a remarkable parallel between this distribution of nuclear alkaline phosphatase and the rate with which the phosphate of thymonucleic acid turns over according to Hevesy and Ottesen. Thus it appears that the role of this enzyme would be to assure the more or less rapid renewal of phosphate of the thymonucleic acid according to the organ considered. This interpretation is further supported by the fact that the alkaline phosphatase reaction increases in intensity in the regenerating liver, where, according to Brues and co-workers, the turnover of phosphate in thymonucleic acid is greatly accelerated.

Let us return, after this rather long digression, to the case of the oöcyte: we see in any case that the facts accumulated up to now do not agree in sustaining the various hypotheses which make the nucleus the center of the oxidations of the egg, a reservoir of enzymes, or the site of protein synthesis. One can regret this negative conclusion, but we have some reasons for hoping that future research

will lead to some fruitful information in the physiological role of the nucleus; thanks to the beautiful techniques now at our disposal, the problem of the chemical composition of the nucleus has become accessible and a vast field is opened to us. Already, new and attractive leads appear, for example, the study of the sulfhydryl proteins which are abundant in the germinal vesicle while the cytoplasm contains very much less. In addition the elimination of classical but unjustifiable hypotheses is progress and at least we have learned how necessary it is to guard oneself against interpretations founded only on microscopic observations for unveiling the functions of structures.

## 2. Spermatogenesis

Without going too far afield, this study will let us mention first an interesting series of investigations devoted to the *cytoplasmic structure* of spermatocytes by Monné; this author has combined the use of the polarization microscope with vital staining by chrysoidine. This leads him to conceive of the basic cytoplasm as composed of two distinct phases: the "spongioplasm" rich in lipids and the "enchylema," more fluid and poor in fats; the latter contains the mitochondria, while the spongioplasm corresponds to the ergastoplasm of the cytologists and is distinguished by its high content of fat and ribonucleic acid. The birefringence of the cytoplasm is principally linked with the lipoidal spongioplasm, while that of the nuclear membrane results from its structure (laminated aggregation of proteins).

As to the structure of the spermatozoön itself, we know that the nucleus gives a strong birefringence with a negative sign. This is due to a parallel orientation of the polymerized thymonucleic acid chains, as shown by Caspersson. The use of the electron microscope has made possible the recognition of some interesting details in the submicroscopic structure of the flagellum; it is made up of 9 to 12 parallel subfibrilles surrounded by a helicord (Harvey and Anderson, Schmitt, Hall and Jakus).

Passing to the *cytochemistry* of spermatogenesis, Caspersson (1939) has studied the amount of *nucleic acids* in the nucleus in a quantitative fashion. The young spermatocytes are relatively poor in substances absorbing ultraviolet at 2650 Å; in the leptotene stage of meiosis, the nuclei become greatly enriched with thymonucleic acid, the concentration of which remains constant during later stages (pachytene and diplotene) of the reduction of the chromatin. Thus

the synthesis of thymonucleic acid precedes the time when the chromosomes become condensed and thickened (pachytene stage); these observations render improbable the idea that the amount of thymonucleic acid is directly responsible for the contraction of the chromosomes; the increase in nucleic acid would be (according to Caspersson) more in relation to the reduplication of the genes.

Brachet (1940, 1941) has gathered some data on the localization of *ribonucleic acid* and *sulfhydryl proteins* during spermatogenesis in insects and amphibians. The nitroprusside reaction is more intense in the primary spermatocytes than in the spermatogonia, without knowing whether the color is purely cytoplasmic or whether the nucleus plays a part. During the mitosis of maturation, the intensity of the reaction decreases in the cytoplasm while the spindle is brightly colored; the chromosomes do not appear to contain —SH groups in appreciable quantities. During spermiogenesis, the color developed in the nucleus increases constantly in intensity; in the mature spermatozoan the head piece (nucleus) gives an exceptionally intense reaction, while the flagellum is notably poor in —SH groups. It is interesting to note that the nuclei of both gametes have an especially high amount of —SH radicals; the significance of this escapes us at the present time. Note also that, at least in certain forms, the nucleus of the mature spermatozoön gives a strong nitroprusside reaction. However, we now tend to hold that these nuclei contain only thymonucleic acid associated with protamines, that is, with the only known proteins free of sulfur. Consider further that the recent analyses of Zittle and O'Dell of total sulfur of methionine, and of cystine of different parts of the bull sperm, isolated by ultrasonics, show that the head is nearly as rich in sulfur as the middle pieces and that it would be valuable to make similar tests on fish spermatozoa which have usually been used to isolate the nucleoprotamines. Pollister and Mirsky have recently confirmed that their nuclei are nearly completely formed of this nucleoprotein. The high amount of basic proteins in sperm heads is also shown by the investigations of Serra and of Thomas, who have ascertained that they give an extremely intense reaction for arginine.

The study of ribonucleic acid shows that the spermatogonia have a cytoplasm much more basophilic than the spermatocytes, while the mature spermatozoa show only a weak coloration of the flagellum; the use of ribonuclease shows that the affinity for basic dyes is due

exclusively to ribonucleic acid. It is noteworthy that cells in mitosis have a cytoplasm with less ribonucleic acid than resting cells. Staining with pyronine-methyl green also reveals some interesting details in the region of the nucleus; that of the spermatids and the spermatocytes stains a bluish green while the heads of the mature spermatozoa appear a clear green; the chromosomes are tinted blue during mitosis; if ribonuclease is used, we notice that the nuclei and the chromosomes now stain clear green whatever the stage in spermatogenesis (Brachet, Matthey). We must conclude from this that the nucleus contains variable quantities of ribonucleic acid associated with thymonucleic acid. At the time of mitosis, the chromosomes contain ribonucleic acid and its concentration in the cytoplasm decreases. The mature spermatozoön contains only traces of this acid. We shall see later on that these cytochemical observations agree perfectly with the chemical findings at our disposal (Chapter VI). They also explain certain observations by Caspersson (1936). This author has shown that absorption in the ultraviolet by spermatozoa is very much less than that of chromosomes of the second maturation division; nevertheless the intensity of the Feulgen reaction does not appear to materially decrease at this time (personal observations). In all probability, the decrease in ultraviolet absorption discovered by Caspersson pertains to the *ribonucleic* acid which is a component of the chromosomes but is lacking in sperm heads. During spermiogenesis there is thus produced a *considerable decrease in the amount of ribonucleic acid in the nucleus and the cytoplasm*, without comparable changes in thymonucleic acid. The reality of this decrease in amount of ribonucleic acid during spermatogenesis had further been established earlier by the determinations of Jorpes and of Brachet (1933). It is interesting to find that a parallel decrease in alkaline phosphatase in the nuclei has been observed by Krugelis, the enzyme finally disappearing in the mature spermatozoa.

We note further that Marza has studied, from a cytochemical point of view, the mature spermatozoa of various mammals. Here are the principal results: the acrosome is formed of proteins, of lecithin and of plasmalogen; the head cap which surrounds the acrosome contains potassium, phosphorus and some lipids; the nucleus contains thymonucleic acid associated with basic proteins (protamines and histones). The anterior centrosome is characterized by great chemical complexity; in fact one finds nucleoproteins, sulfur,

lecithin, and potassium. The posterior half of the head piece is covered with a "lipoidal mantle" containing, besides lecithin, some proteins, P, S, and K. Finally, the neck and flagellum are formed by proteins, while in the middle pieces one finds proteins, glycogen, and lecithin. The results of Marza have since been confirmed by Kirpichnikova, who has shown further that, although calcium and magnesium predominate in the nucleus, sodium and potassium are chiefly encountered in the flagellum.

The distribution of the haemins and cytochrome oxidase has been studied by Zittle and Zitin: these components of the respiratory system of the cell are abundant in the tail but exist in only small quantities in the head; the collar occupies an intermediate position in this respect.

We come to consider now investigation pertinent to the *metabolism of the sperm*. Having in mind the analogies between sperm motility and muscular contraction, we ask whether there is a corresponding similarity in metabolism; numerous researches (Meyerhof, Parnas, etc.) have demonstrated that glycolysis (transformation of glycogen into lactic acid) and oxidation of the carbohydrates are the principal energy sources in muscle. Is it the same in spermatozoa? Most of the recent works carried out with the mammals answer this question in the affirmative (Redenz, Iwanow, Torres, Comstock, MacLeod, Lardy, Phillips, and Mann). In fact it has been found that the sperm remain motile under anaerobic conditions if one adds a utilizable sugar; thus glycolysis may become an energy source when respiration is inhibited; in the presence of oxygen both respiration and sperm motility are stimulated by the addition of glucose (Redenz). Probably glucose is anaerobically transformed into lactic acid and this is then rapidly oxidized. The addition of lactate or pyruvate increases the oxygen consumption without, however, stimulating sperm motility; from this Redenz concludes that glycolysis is the principal energy source both under aerobic and anaerobic conditions. However, Iwanow has established that spermatozoa remain motile in the presence of  $O_2$  when monoiodoacetic acid is added and this acid specifically inhibits glycolysis; this poison stops movement very quickly under anaerobic conditions. Iwanow concludes that the spermatozoa under anaerobic conditions draw upon glycolysis for the energy necessary for motility, while in the presence of oxygen they use the energy from the oxidation of various

substrates; the fact that the addition of pyruvate (Iwanow 1940) maintains movements under anaerobiosis confirms the importance of glycolysis under these conditions. Some investigations designed to see whether the sperm, like muscle, can synthesize phosphocreatine at the expense of creatine and phosphoric acid have given contradictory results (Torres, Iwanow, 1937).

Certain observations by Winberg on the metabolism of rooster sperm also speak in favor of a carbohydrate metabolism. In fact the respiratory quotient is 0.92, which is close to that characteristic of the oxidation of carbohydrates (1.00); these spermatozoa easily oxidize glucose, fructose, and mannose and, with more difficulty, maltose; they cannot use glycogen, sucrose, lactose, galactose, etc. The addition of oxidizable sugars is accompanied by an appreciable lactic acid production (aerobic glycolysis). Finally, Winberg (1941) has reported that rooster sperm contains large amounts of cozymase, *i.e.*, the coenzyme in glycolysis.

More recently, T. Mann has clearly established that spermatozoa possess a carbohydrate metabolism closely resembling that of yeast and muscle. Thus we are dealing with a glycolysis with phosphorylations at the expense of adenosine triphosphate; this latter can be isolated from sperm and it is broken down under anaerobic conditions in the absence of glucose. It takes part in the phosphorylation of glucose and hexosemonophosphates. These are interesting observations because they have been made on intact spermatozoa, while our knowledge of glycolysis in yeast and muscle comes from studies on purified extracts. Thus the process of glycolysis in the living cell is much as we supposed from research on extracts.

In addition, Mann has been able to detect in mammalian spermatozoa cytochromes, a, b, and c, and he has found large amounts of iron, copper, and zinc. Thus the sperm is a cell with a high metabolism which obtains the energy necessary for motility by means of glycolysis, using fructose, which Mann (1946) has found in abundance in the seminal fluid.

In the marine invertebrates, anaerobiosis rapidly arrests sperm movements (Barron); the sperm are provided with oxidative enzymes and carriers of hydrogen (cytochrome oxidase, cytochromes a, b, c, and succinic dehydrogenase) according to Ball and B. Meyerhof and Krahle, Keltch and Clowes—all working on the sea urchin, *Arbacia*. Although Lindahl has not detected cytochrome in the

sperm of another sea urchin, *Paracentrotus*, Brachet has found its spectrum in frog sperm (1934). More recently, Barron and Goldlinger have noted that the sperm of *Arbacia* oxidize pyruvate,  $\alpha$ -ketoglutarate, succinate and *l*(+)-glutamate; these sperm thus have the same activity as muscle, which also oxidizes these compounds.

Examination of the recent literature allows us to conclude that the *mature spermatozoa have a high respiratory metabolism and that they utilize, in the main, carbohydrates as an energy source.*

### 3. Conclusions

The oöcyte during its growth phases is evidently the site of particularly active synthetic processes; this opinion is confirmed by cytochemical examination. Unfortunately, we still do not know completely the chemical mechanism of these syntheses; however, one may assume, without danger of too great error that the enzymes which hydrolyze the proteins, fats, and sugars also bring about their synthesis. The observations of Duspiva on the dipeptidase activity of the frogs' oöcyte confirm this view. It would certainly be valuable to investigate the enzymes in the oöcytes which we know catalyze syntheses as well as hydrolysis: we think chiefly of the hexosephosphate (Cori's ester) in muscle and in potato. The nuclear and cytoplasmic localization of such an enzyme, its variations in concentration during oögenesis, would surely merit intensive study.

The most suggestive results obtained up to the present are without doubt those which concern the localization of enzymes in the oöcyte. We know now that the oxidizing enzymes (desmolases) are for the most part bound to granules while most of the hydrolases are found in the hyaloplasm; it may be, of course, that the hydrolases are also bound to particles but these may be too small to be thrown down by the centrifugal forces used; we shall return to this point in Chapter VI. In addition we have rid ourselves of the old myths of "nucleus as center of oxidations" and of "nucleus as site of accumulation of enzymes." We have learned to suspect physiological interpretation based on purely descriptive observations. The hypothesis which attributes to the nucleus an important role in syntheses does not rest on firm ground; however, it would be premature to reject it definitely and to accord to the nucleus merely an accessory role. The recent biological observations show that enucleated fragments of *Arbacia* eggs (E. B. Harvey) and of the

unicellular alga *Acetabularia* (Hämmerling) survive for weeks even months: this cytoplasm without nucleus ought certainly to respire and perhaps assimilate; its biochemical study would be of the greatest interest. But what chiefly characterizes these enucleated fragments is their inability to undergo morphogenesis, whether it be regeneration or embryonic development. Thus these processes are under the influence of the nucleus which "induces" morphogenesis as has been proved by Hämmerling's experiments; it is probable that the nucleus is the site of the synthesis of specific substances elaborated by the genes; these substances are not necessarily present in large quantity and thus they do not imply a particularly high concentration of enzymes in the nucleus; their importance is nonetheless very great. We will have occasion to discuss this later on.

As to the spermatozoön, its metabolism corresponds very well to what we might have predicted: this very active cell has an intense metabolism and the maintenance of this latter is indispensable to the continuance of motility. This metabolism is directly comparable to that of muscle. Thus one finds once more, on the biochemical level, the basic analogies between ciliary movement and muscular contraction which general physiology has brought to light.





## CHAPTER IV

# Biochemistry of Fertilization

Let us review briefly the various stages in the process of fertilization. Fertilizable eggs of various species are found to be in different stages of maturation. The eggs of some species are surrounded by membranes formed during the passage through the genital tract of the female. The spermatozoön is attracted by the egg, comes in contact with it, and a "cortical reaction" ensues. The ultramicroscopic structure of the outer layer of the egg (cortex) is profoundly modified as a wave of contraction proceeds from the point of sperm attachment over the surface of the egg. The "fertilization membrane" lifts off the surface of the egg which is now separated from the membrane by a layer of liquid, the perivitelline fluid filling the perivitelline space. This contraction of the egg reduces its diameter to some degree. The spermatozoön now penetrates the egg, which completes its maturation and becomes an oötid. Around the head of the spermatozoön a large aster (sperm aster) develops, and the sperm nucleus then migrates, with the sperm aster, toward the nucleus of the oötid (female pronucleus). At the same time, the male nucleus swells and loses its compact form (transformation into the sperm pronucleus). The sperm aster withdraws and the two pronuclei come together and finally fuse (amphimixis). Two asters now appear, heralding the first mitosis, which commences the next phase of development (segmentation).

The mechanism of the elevation of the fertilization membrane has recently been analyzed using the polarization microscope by J. Runnström, L. Monné and L. Broman, and Runnström, Monné, and Wicklund. With this instrument, the cortex is seen to be formed of elongated molecules arranged at random with respect to the surface. This cortical layer, which is isotropic, liquifies at the time of pene-

tration of the spermatozoön. The elongated molecules orient to form birefringent lamellae, which make up the fertilization membrane. The solidification of this membrane is correlated with the formation of —SS—linkages.

Fertilization, which releases the unfertilized egg from an inactive state, has been the object of numerous biochemical studies. The attraction of the spermatozoa by the egg has been especially examined, as have the changes in metabolism which follow fertilization. The essential role of the sperm can be analyzed by comparing fertilization with artificial parthenogenesis, in which development of the egg is stimulated by physical or chemical means.

### 1. Chemical Nature of "Fertilizin." Gyno- and Androgamones

#### *a. Gynogamones*

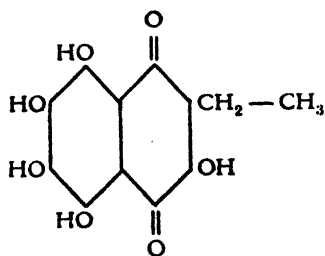
The effect of egg secretions on the spermatozoön was recognized many years ago by von Dungern and by J. De Meyer; to an American biologist, F. R. Lillie, belongs the credit of having carefully analyzed the phenomenon and of having drawn interesting deductions concerning the mechanism of fertilization. He showed that sea water containing the eggs of the sea urchin ("egg water") had a threefold effect on the spermatozoa. (1) An *activation*, that is a stimulation of movement. Immotile sperm can regain motility if egg water is added, this activation being accompanied by an increase in respiration which may amount to 500% (Tyler). (2) A *positive chemotaxis*, which means that the sperm are attracted by egg secretions. (3) *Agglutination*, in other words an aggregation of sperm in clumps of various sizes. This agglutination is reversible if the action of the egg water is not prolonged beyond a certain time. It is this factor for agglutination (agglutinin) to which Lillie attributes the most importance. It is responsible for the attraction of the spermatozoa and is thus given the name "fertilizin." Most of the biologists who have repeated the research of Lillie and his school have criticized these experiments and cast strong doubt on the physiological role of fertilizin (see, in this connection, A. Dalcq's book (1928), in which a discussion of this question will be found).

After a period of relative obscurity, the problem of fertilizin again attracted the interest of biologists. It has been especially studied in Germany by Hartmann and Schartau, with the collabora-

tion of R. Kuhn and Wallenfels on the chemical aspects, in the United States by Tyler and Fox and Cornman, and in Sweden by J. Runnström and his collaborators. We will first examine the results obtained by Hartmann's school and then see how well they agree with the results obtained almost simultaneously by the American authors.

Hartmann and Schartau have verified, first of all, for the sea urchin egg the chemotactic and activating effects of egg water on the sperm. If one introduces into a drop of sperm a capillary containing egg water, one notices that the sperm penetrate rapidly into the capillary; they are not attracted by a pipette filled with sea water, which serves as a control. Microscopic examination shows that the egg water accelerates sperm movement; hence, the presence of substances influencing the sperm in the water in which eggs have been kept is confirmed. It may be asked next, what is the nature of these substances and what is their localization within the egg? Lillie had already noted that fertilizin resists brief boiling and that it is found in abundance in the mucous jelly surrounding the sea urchin egg. Hartmann and Schartau have gone a step further in showing that one can dissociate, by the action of heat or light, the "agglutinating" factor from the "activating" factor in the egg water. Exposure to bright light causes the activation and chemotactic properties of egg water to disappear without affecting its agglutinative power. Heating for two hours at 95°, on the contrary, destroys the agglutinating factor selectively.

Hartmann and Schartau, in collaboration with Kuhn and Wallenfels, have shown that the factor responsible for activation and chemotaxis is to all appearances none other than *echinochrome A*, that is to say, the bluish-red pigment of the eggs. This substance is a derivative of naphthoquinone and has for its formula:



Echinochrome A thus will stimulate sperm movements and will attract them at the enormous dilution of 1 part in 2,500,000,000 parts of water. Light rapidly inactivates it, but it resists boiling. Hartmann and his collaborators called it "*gynogamone I.*" Agglutination is brought about by a second factor "*gynogamone II.*"

Later work of Kuhn and Wallenfels (1940) has shown that echinochrome A is not present in the free state in egg water. It enters into the formation of a complex with a protein or, more accurately, it conjugates with a protein. The existence of this conjugated protein in the jelly of eggs had been suspected by Glaser and Lederer, who were the first to isolate echinochrome. The complex compound present in jelly may be either binary or tertiary. In the first case, echinochrome A is combined with a protein; in the second case, another component is added (*Hilfsträger*). Jelly-less eggs give only the binary compound; thus the jelly provides the third constituent. The latter has been partially purified, and is a substance similar to proteins, containing 8 to 8.5% of nitrogen. The tertiary compound is precipitated by ammonium sulfate, and, if the product obtained is dialyzed, the third component (*Hilfsträger*) separates out and the denatured binary compound (echinochrome-protein) is precipitated.

The biological study of this substance has shown (Hartmann, Kuhn, Schartau, and Wallenfels) that the binary compound is completely inactive; the ternary compound, on the contrary, is very effective in agglutination, in activation, and in chemotaxis. It is only necessary to add *Hilfsträger* to the binary compound to activate it. As we have seen, *Hilfsträger* is localized in the jelly, which also contains agglutinin: it might be expected that the two substances are identical; as a matter of fact, the experiments show that purified *Hilfsträger* has a strong agglutinating action on spermatozoa. The active substance in egg water would thus be the ternary compound, with echinochrome A playing the role of gynogamone I, while *Hilfsträger* would be identical with gynogamone II (agglutinin).

Finally, Runnström and Monné have reported an egg substance which acts on eggs placed in hypertonic sea water. In the presence of this substance, the "wrinkles" that appear as an effect of hypertonicity disappear and the surface of the egg becomes smooth; this "smoothing" of the surface is retarded in the absence of calcium. It seems that this substance tends to bring about the liquefaction of the egg cortex, and that it plays an important role in activation.

Other investigators who worked on some different species attempted to verify this work. Tyler became interested in echinochrome because Friedheim had attributed to this pigment the ability of increasing the respiration of unfertilized sea urchin eggs 16-fold. Tyler isolated the pigment but did not confirm Friedheim's observations. In the meantime, the work of Hartmann and his collaborators having been published, Tyler examined the effects of crystallized echinochrome on the respiration, motility, and agglutination of sperm. He used the sperm of a different species of sea urchin (*Strongylocentrotus* instead of *Arbacia*) and he obtained negative results. Oxygen consumption, motility, and agglutination of these spermatozoa were not modified in any way by the addition of echinochrome. The reason for these contradictions is not apparent. Tyler noted that *Strongylocentrotus*, in contrast to *Arbacia*, does not contain echinochrome, while Schartau (1940) suggested that the echinochrome prepared by Tyler differs from that isolated by Kuhn and Wallenfels. However, the method of preparation and the melting point values argue for the identity of the two substances. Be that as it may, the egg water of *Strongylocentrotus* definitely activates the sperm of this species, so that we must conclude that echinochrome has only a limited role.

The question has been taken up by Cornman (1941), who investigated the effects of echinochrome on the American species of *Arbacia*, *A. punctulata* instead of *A. pustulosa*, the species used by the German workers. He noted no effect whatever on the sperm when echinochrome was added to them, although he used sea urchins with an abundance of pigment. Struck by the fact that these sperm reacted strongly to changes in pH, Cornman raised the question of whether the chemotaxis of Hartman and Schartau is not caused by the greater alkalinity of the capillary containing echinochrome as compared with that filled with sea water. This source of error does not appear to have been foreseen by Hartmann and Schartau. Cornman emphasized the fact that echinochrome in a buffered solution does not stimulate chemotaxis even in high concentrations. However, the American author confirmed the activity of the conjugated protein and obtained, by the method of Kuhn and Wallenfels, a protein colored by echinochrome, which activated and agglutinated sperm. Dialysis of this extract for 20 minutes permits the separation of these two gynogamones. Cornman, however, attributed no role whatever to echinochrome. It is further known that egg water

of colored sea urchin species activates the sperm of species lacking the pigment and *vice versa* (Woodward). Cornman completed his interesting paper with a review of the earlier observations on the chemical nature of fertilizin. Glaser (1921) noted that a lipase prepared from eggs or from pancreas activated sperm, while Clowes and Bachman obtained a gynogamone I by distillations of the sperm, an observation confirmed by Cornman. This distillate can be replaced by the higher alcohols (propyl, allyl, cinnamyl). Cornman thus proposed the following hypothesis: The active agent is a higher alcohol that liberates a nondialyzable lipase; the former would be the gynogamone I of Hartmann, the latter would accompany agglutination (gynogamone II).

We owe to Tyler and to Fox and Tyler (1941) a contribution to the study of the agglutinin present in egg water of a mollusk (*Megathura crenulata*) and the sea urchin *Strongylocentrotus purpuratus*. This agglutinin is, in both cases, precipitated by ammonium sulfate and is nondialyzable. It gives the color reactions characteristic of the proteins and is completely inactivated by crystalline trypsin or chymotrypsin. The protein nature of this agglutinin thus appears very probable, but the purest preparations contain only 5 percent nitrogen and thus can not be composed exclusively of proteins. According to Tyler, the agglutinin of egg water, like that described by immunologists, is a protein, and the mode of action of the two substances is probably similar.

Tyler (1940, 1942) has made the interesting observation that when one attempts to extract large quantities of fertilizin by breaking up the eggs, the yield is much less than that obtained from egg water. It must be concluded that the fertilizin, which is in the jelly surrounding the egg, is neutralized by an antifertilizin present in the interior of the egg. This substance is really an antibody against the fertilizin of the egg surface. The presence of these two complementary substances has led Tyler (1942) to formulate an original hypothesis concerning cellular structures. Briefly, he supposes that the cell may be a mosaic of substances capable of combining one with another in the manner of an antibody with an antigen, the combination taking place at the points where these substances are adjacent. The products of this combination may be represented by the various membranes (cellular, nuclear, nucleolar, vacuolar), the role of which may be to keep the various complementary substances separate.

A point that may influence research on the chemical nature of the gamones is that the addition of traces of copper prevents fertilization, probably by reason of a combination with fertilizin (Lillie, Runnström). Since copper combines with the —SH groups, Townsend thought that glutathione may be one of the gamones; this supposition is based chiefly on the ability of this tripeptide (abundant in sperm) to activate the movement of spermatozoa. But Runnström has stated that monoiodoacetic acid, which combines specifically with —SH groups, does not prevent fertilization; and he believes that copper may combine in the cortex of the egg, explaining why, when eggs are shaken, they are much less sensitive to the action of copper, since shaking favors the release of fertilizin into sea water—where it combines with the copper. Runnström considers fertilizin as a thermostabile coenzyme which may combine with proteins of the egg to form the complete enzyme.

It is clear that research in this field is complicated by the fact that a number of agents activate the movements of sperm (alkalinity, glutathione, echinochrome (?), etc.). Information about gynogamone I may thus be hoped for only when it is isolated from egg water. Some results have already been obtained along this line. The presence of gynogamones identical with the fertilizin of Lillie, is now beyond doubt. It is well established, furthermore, that these substances are present in the jelly and that the agglutinin can be separated from the activating agent. There is strong evidence in favor of the protein nature of agglutinin, while the sperm-activating principle is probably a substance with a small molecule, its identity with echinochrome being doubtful at the present time. In fact, this latter substance is without action in certain species of *Arbacia*. These gynogamones are probably not indispensable for fertilization, since this process can take place in eggs from which the jelly has been removed. The gamones thus constitute merely useful adjuncts which facilitate the penetration of the egg by the sperm.

#### *b. Androgamones*

It may be asked if the production of substances favoring fertilization is a property exclusive to the mature egg. Only recently has systematic research begun to see if the spermatozoa also release gamones into the water. Southwick noted in 1939 that if one centrifuges concentrated sea urchin sperm, the supernatant liquid *inhibits*



the motility of sperm; this liquid also has the property of neutralizing the agglutinin (gynogamone II) present in egg water. If one mixes the sperm liquid with egg water the former loses its capacity to inhibit sperm movement.

Frank, also in 1939, studied extracts of spermatozoa of the sea urchin prepared by heating in sea water. These extracts *agglutinated* eggs of the same species with or without the presence of the jelly. This agglutinin for eggs is only found in the spermatozoa. It is a colorless substance, colloidal, nondialyzable, and resistant to boiling; it gives none of the color reactions of proteins, is not precipitated by ammonium sulfate, and is insoluble in an ether-alcohol mixture. These extracts of Frank neutralized the chief activator (gynogamone II) present in egg water. They do not stimulate development of the unfertilized egg, but, on the contrary, exert an inhibiting effect on fertilization and on the development of normally fertilized eggs.

Tyler (1940) resumed the study of the substance discovered by Frank, taking care to freeze the spermatozoa at very low temperatures before treating them with warm sea water. This freezing gave a better extraction, and Tyler obtained, in this way, very active preparations which were precipitated by ammonium sulfate. They gave protein reactions very clearly and it appears that the failure of Frank to obtain these reactions was due to the excessive dilution of his extracts. This agglutinin against eggs appears to be similar to the agglutinin against sperm (gynogamone II) present in egg water. Further, Tyler (1939) isolated from the sperm of *Megathura crenulata*, by freezing them at  $-80^{\circ}\text{C}$ . and quickly thawing them, a substance that dissolves the membrane surrounding the egg. This *lysin* is a protein, since its activity is lost after digestion with crystalline trypsin or with chymotrypsin. A lysin is also found in another mollusk, *Haliotis*. These two substances are specific in the sense that they only dissolve the membranes of eggs of the same species.

The research of Hartmann, Schartau, and Wallenfels on the gamones of the sperm of *Arbacia* has helped to clarify the question. The supernatant liquid from centrifugation of the concentrated sperm exerts four distinct actions: (1) paralysis of the sperm (Southwick's substance); (2) neutralization of gynogamone I (which activates the movement of the spermatozoa); (3) lysis of the jelly (lysin of Tyler); (4) neutralization of gynogamone II (agglutinin against spermatozoa). Hartmann, Kuhn, Schartau, and Wallenfels were

able to show that there are really only two substances present that exert a two-fold effect. Androgamone I paralyzes the movement of sperm and neutralizes gynogamone I, while androgamone II dissolves the mucous jelly and neutralizes gynogamone II. The two androgamones are easily separated, androgamone I being soluble in methanol. One can isolate from methanol extracts a colorless substance which inhibits sperm motility in a concentration of 8–9 mg/cc.

Androgamone II is a yellow substance which is very soluble in sea water and which, even at a concentration of 0.4 mg/cc., rapidly dissolves the jelly. Both androgamones are resistant to boiling.

It is interesting to note that the addition of a crystal of echinochrome A is sufficient to completely inhibit the paralyzing action of androgamone I on spermatozoa. There must be a chemical reaction between these two substances, since one can detect an immediate change in the absorption spectrum of echinochrome.

Hartmann attributes the following physiological roles to these gamones: gynogamone I, which activates and attracts spermatozoa, evidently facilitates fertilization; androgamone I, an antagonist, tends to paralyze spermatozoa, thus prolonging their fertilizing power. The success of fertilization thus depends on the equilibrium between these two gamones. The agglutination of sperm by gynogamone II is produced only by large doses of the substance and probably has no physiological importance. This agglutination is a consequence of a change in the surface layer of the spermatozoa, and perhaps this change ensures the fusion of the gametes. Androgamone II naturally favors the penetration of the egg by the spermatozoön by dissolving the protecting jelly. The gamones also probably play a role in preventing hybridization, since gynogamone II strongly agglutinates sperm of foreign species (heteroagglutination), and it is established that hybridization is facilitated by suppressing this agglutination.

The gamones are not found exclusively in the sea urchin. Montalenti and Schartau have found two gynogamones and two androgamones which antagonize each other in the lamprey. Their physiological properties are the same as the analogous substances of the sea urchin. von Medem has observed some similar phenomena in a number of marine mollusks.

Finally let us mention a recent study by Runnström, Tiselius, and Vasseur on the androgamone II of the echinoderm, *Echinocardium cordatum*. According to the concentration used, this androgamone

first stimulates swelling, then agglutination, and finally precipitation of the jelly surrounding the eggs of *Psammechinus miliaris*. Analogous effects are obtained with serum or with extracts of organs of fish or with bee venom. The dialyzed extract of sperm contains a chief constituent having an acid reaction and a molecular weight between 2,000 and 10,000, and, in addition, a derivative of nucleic acid, judging from the absorption spectrum and the presence of phosphorus. Finally, one finds a lecithinase which transforms lecithin into lysocithin, which is able to lyse red blood corpuscles. It is assumed that this enzyme takes part in the precipitation and agglutination of the jelly. Still more recently, Runnström, Lindvall, and Tiselius have shown that androgamone II is rapidly inactivated by trypsin; it is thus probably a protein. The same authors have evidence for an androgamone III that liquefies the cortex. This is a substance that is soluble in methanol, dialyzable, resistant to trypsin and different from androgamone I.

This group of studies indicates that the question of sperm and egg secretions, after a long eclipse, is again being actively investigated. The physiological role of the gamones and their mutual antagonism are now well established, and the problem is now ready for chemical analysis, which has already furnished us with a few well established facts.

## 2. Effects of Fertilization on Metabolism of the Egg.

### General Comments

The variations of metabolism, especially those of respiration, which follow fertilization, have been the object of extensive investigations. We shall limit the discussion of the general phases of the problem in order to examine in detail the special case of the sea urchin egg, which has provided the material for a particularly large number of experiments.

It was in 1908 that Warburg noted that fertilization produced in the sea urchin egg a rapid and large increase in the rate of oxidation. He established that the rate increased fivefold in the space of a few minutes. This fundamental discovery was later confirmed by a number of workers (notably Shearer, Runnström, Whitaker, Laser, and Rothschild, etc.) working on a variety of sea urchins. The conclusions of Warburg had an enormous influence, because they provided striking confirmation of Loeb's theory (1906) of the chemical mechanism of fertilization. Loeb held that the spermatozoön or

parthenogenetic agents acted by modifying the surface of the egg, resulting in an increase in oxidations which led to cytolysis if it was not corrected by an "immunization" of the egg.

But it is important in the first place to know if this phenomenon is of universal occurrence. Without leaving the phylum *Echinodermata*, it may be noted that the results in the starfish egg are vari-

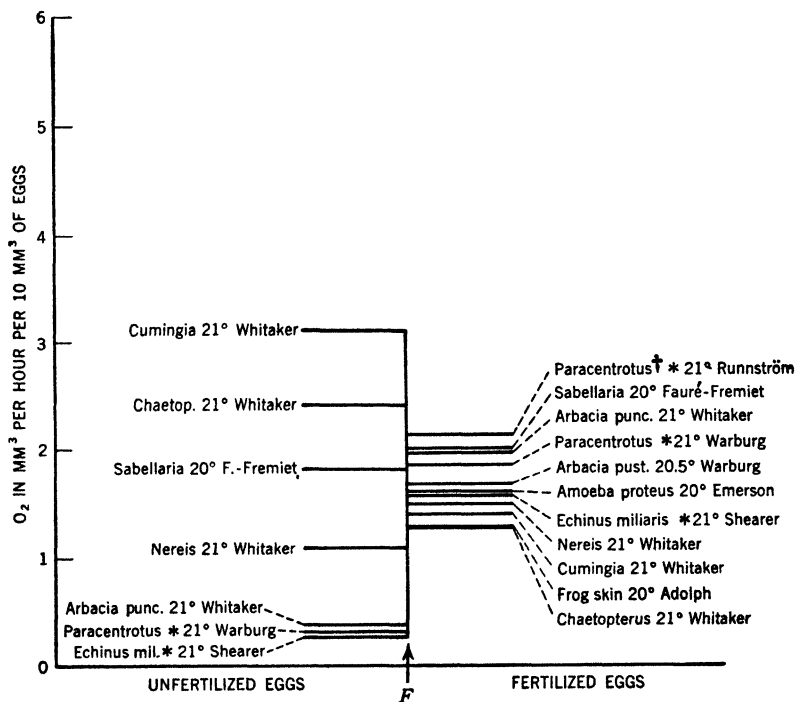


Fig. 21. Effects of fertilization on oxidation in various marine invertebrate eggs (Whitaker).

\* Where the temperature is preceded by an asterisk, a temperature correction has been made. † This figure would probably have been lower if the measurement had been limited to the first hour after fertilization.

able. While Loeb and Wasteneys and P. S. Tang (1931) observed no changes in respiration during fertilization in *Asterias forbesii*, Borei has noted, on the contrary in *Asterias glacialis* eggs an increase in oxidation as marked as that in the sea urchin egg. Recently, Shapiro (1941) reexamined the problem in *Asterias forbesii* and noted a large variability from one batch of eggs to the other. While some

lots of eggs behaved in conformity with the ideas of Loeb and Wasteneys and Tang, most of them showed a substantial increase in oxidation following fertilization. This variability leads one to believe that the increase in respiration discovered by Warburg may not possess the great importance first attributed to it.

When we go afield from phylum *Echinodermata*, we soon see that the phenomenon noted by Warburg is not at all general. In a worm (*Sabellaria*), Fauré-Fremiet (1924) found no difference in metabolism between fertilized and unfertilized eggs. In the egg of another species of worm, *Nereis*, fertilization causes only a slight increase (25%) in the rate of gaseous exchange (Barron, Whitaker). In the ascidian, *Ciona*, on the contrary there occurs an appreciable increase in the oxygen consumption of the egg (of the order of 170%) after fertilization (Runnström, Tyler and Humason). In the echiurid worm, *Urechis caupo*, Tyler and Humason obtained results similar to those of Shapiro on the starfish *Asterias forbesii*. According to the particular batch of eggs, respiration increased, was not changed, or even decreased at fertilization.

It was the work of Whitaker that clarified this confused situation. He studied the effect of fertilization on eggs of a series of marine invertebrates (*Cumingia*, a lamellibranch; *Nereis* and *Chaetopterus*, polychaete worms; *Arbacia*, an echinoderm). The values vary greatly from one species to another as shown by figure 21. Instead of an increase in respiration, we find in the egg of *Chaetopterus* a decrease which reaches 50%. This fact has since been confirmed by Chesley, Shapiro (1936), and Brachet (1937, 1938).

The rate of oxygen consumption expressed as a function of the same volume of eggs varies in the manner illustrated in Figure 21. Unfertilized eggs of different species respire at vastly different rates, but fertilization brings these rates closer together. A fertilized egg of *Arbacia* for example, respire, at equal volume, like that of *Chaetopterus*, although the rates of oxidation of the unfertilized eggs may differ by a proportion of 1 to 5 or 6. It is thus incorrect to say, as has been said so often, that fertilization increases the respiration of the egg. In reality, it *regulates* the metabolic exchange. What is abnormal is the excessively low respiration of the unfertilized sea urchin egg and the very high rate of oxidation in the unfertilized *Chaetopterus* egg, and not the increase in respiration in the former or the decrease in the latter.

Whitaker has cogently remarked that the "normal rate" characteristic of the fertilized egg is about that which is exhibited by most cells in a state of growth. It is understood, of course, that one would not expect that this relationship established by Whitaker would hold for very large eggs with a cytoplasm filled with yolk.

Let us point out in passing that the higher respiration of unfertilized eggs of *Chaetopterus* and *Cumingia* is not due to the fact that they are in a different stage of maturation from that of the sea urchin egg (metaphase and oötid, respectively). In fact, the unfertilized egg of *Ciona* is also arrested at the metaphase of the first maturation division and, as we have seen, the respiration of this egg rises at fertilization.

The principal conclusions of Whitaker can be summarized as follows. The cause of the inertia of the unfertilized egg is probably not directly related to the intensity of its oxidation, and the stimulus for development is not necessarily accompanied by an increase in respiration. The case of the sea urchin egg is a special one and does not afford evidence for deductions of a general nature. Loeb's theory, which is applicable to the sea urchin egg does not hold for other species. The real problem is to understand the causes of the inhibition of the unfertilized egg and the reasons for its abnormal metabolism. Whitaker makes a comparison between narcotized cells and the unfertilized egg, mainly from the point of view of their viscosity, permeability, and oxidations. The aberrant metabolism of unfertilized eggs of the sea urchin and of *Chaetopterus* is probably concerned with their inertia. However, there is no evidence to show that a causal relation exists between the two phenomena.

Whitaker's work has certainly contributed toward the clarification of the problem and we believe his conclusions are justifiable. Furthermore, one finds in the recent literature arguments favoring his ideas, as for example, the observations of Tyler and Humason on variations in respiration at fertilization in the egg of *Urechis*. It was observed, in this case, that according to the batch of eggs the rate of oxidation increases, remains constant, or decreases. But these changes depend essentially on the intensity of the metabolism of unfertilized eggs (if it is high, fertilization decreases respiration), which appears once more to be abnormal. The research of Lindahl and Holter on the sea urchin egg (see later) also provides evidence for a progressive inhibition of oxidation during the course of maturation. The idea that the respiration of unfertilized eggs is frequently

abnormal and that it is regulated by fertilization thus seems to have a solid foundation.

Let us now examine the case of eggs with a large amount of cytoplasm, rich in yolk. In the frog egg, Bialaszewicz and Bledowski, and later Parnas and Krasinska, found an increase in the gaseous exchange at fertilization. Later research (J. Brachet, 1934; Stefanelli, 1938) carried out with more sensitive instruments, permitted measurements at close intervals. The results definitely precluded the idea of changes in the rate of oxygen consumption at the time of fertilization in the eggs of the amphibians studied (frog, toad) (Fig. 22). The writer, however, has found that fertilization produces a

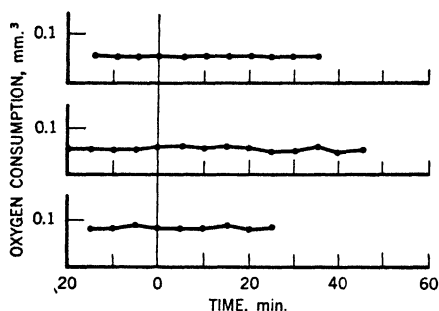


Fig. 22. Oxygen consumption in toad egg during fertilization (Stefanelli).

profound change in the *respiratory quotient* (R.Q.), which goes from 0.99 to 0.66. This drop in R.Q. shows that if fertilization does not change the rate of oxygen consumption, it does influence its character. The unfertilized egg probably utilizes carbohydrates, while the fertilized egg is the site of incomplete oxidation, as indicated by the abnormally low respiratory quotient. Since the measurement of this quotient takes considerable time (10–15 hours), one can not decide whether the metabolism changes abruptly at the time of fertilization or whether the change is slow. A few indications tend to show that the case of the frog's egg is not unique in this respect. Horowitz (1940) has found that the R.Q. of the eggs of *Urechis* goes from 0.69 to 0.89 or 1 after fertilization. In the sea urchin, on the contrary, Laser and Rothschild have obtained an extremely low value a few minutes after insemination. It may be that *qualitative* differences in metabolism are more frequent during fertilization than has been hitherto supposed. This should probably be considered as an indication of abnormal metabolism of the egg, in connection with its inertia.

Let us mention one more point of interest concerning the factors responsible for the inactivity of the unfertilized egg. Bataillon and Tchou-Su (1929, 1930) have shown that the anuran egg is not fertilizable if it has remained too long in the uterus; to explain this, they formulated the hypothesis that the egg in passing thru the female genital tract is anaesthetized by  $\text{CO}_2$ . Some ingenious experiments led them to conclude that eggs taken from the oviduct become fertilizable if the  $\text{CO}_2$  they contain is removed by placing them in a chamber over sodium hydroxide. Furthermore mature uterine eggs lose their fertilizability when they are treated with  $\text{CO}_2$ . Since, according to the experiments of Bialaszewicz and Bledowski, frogs'

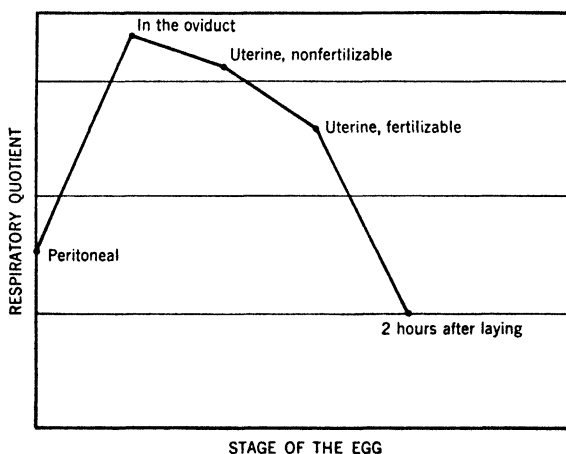


Fig. 23. Amount of  $\text{CO}_2$  in unfertilized frog egg taken from different regions of the genital tract (Dalcq, Pasteels, and Brachet).

eggs give off large quantities of  $\text{CO}_2$  immediately after leaving the uterus, Bataillon and Tchou-Su were justified in believing that intoxication by  $\text{CO}_2$  was, in this case, the physiological cause of the inactivity of the unfertilized egg. More precise measurements made by Brachet, in collaboration with Dalcq and Pasteels, have confirmed this interpretation. If this interpretation is correct, the  $\text{CO}_2$  content of immature eggs ought to be higher than that of mature eggs removed after they remained some time in the uterus. Dalcq, Pasteels, and Brachet have measured the respiratory quotient of eggs from the body cavity, from the oviduct, and from the uterus. In



the latter case the eggs that had just arrived in the uterus were compared with those that had been in the uterus for one or two days and were normally ready for fertilization.

It is evident that there is no question here of a true R.Q., since besides the  $\text{CO}_2$  produced by the egg, that which was dissolved in the jelly and the egg was also included in the measurement. Figure 23 shows clearly that, in conformity with the idea of Bataillon and Tchou-Su, eggs situated in the oviduct and those that have just entered the uterus have a higher  $\text{CO}_2$  content than eggs that have been in the uterus for some time and are fertilizable. Body cavity eggs can not be fertilized, perhaps because of the lack of the mucous jelly.

The existence of differences between eggs taken from successive regions of the genital tract was corroborated by Stefanelli (1938). In the toad (*Bufo vulgaris*), the oxygen consumption goes from  $0.0043 \text{ mm}^3$  in four minutes per egg for oöcytes in the ovary to  $0.005\text{--}0.006 \text{ mm}^3$  for eggs in the body cavity, and to  $0.0082 \text{ mm}^3$  for eggs located in the upper part of the uterus; it finally reaches  $0.0093 \text{ mm}^3$  for ripe fertilizable eggs. Thus, a regular increase in respiration takes place during the passage of the egg through the female genital tract.

It will be remembered that fertilization does not modify the rate of oxygen consumption in the toad egg. In the lamprey egg, however, the situation is different (Stefanelli). The respiration of unfertilized eggs is about  $0.0132 \text{ mm}^3$  per egg for 5 minutes while a value of  $0.0277$  to  $0.033 \text{ mm}^3$  is attained after fertilization. Recent research of Phillips (1940) has established that, contrary to the earlier results of Hyman and of Boyd, fertilization does not change oxygen consumption in the eggs of the marine teleost fish, *Fundulus heteroclitus*.

What conclusions are we to draw from all these observations? Fertilization often alters the respiration of the egg, but in an extremely variable manner. The sudden increase upon fertilization, which characterizes the respiration of the sea urchin egg, is opposed to the marked inhibition in the egg of *Chaetopterus* under the same circumstances. Even in the cases in which the rate of respiration is not modified, it may be that the nature of the oxidized substrates changes, as is indicated by observations on the frog's egg. Before making a definite judgment, it would be necessary to be certain that

the R.Q. of eggs in which oxygen consumption remains constant at fertilization shows no change. Everything considered, we may suppose that fertilization exerts an influence on the degree and the nature of the oxidations, but that the changes which it brings about are understandable only if we agree with Whitaker that the metabolism of the unfertilized egg is frequently faulty. Such a conclusion is in good agreement with all the current ideas on the physiology of the unfertilized egg, which appears to be an egg with its permeability decreased, and in a more or less profound state of inhibition. In the frog's egg the retention of  $\text{CO}_2$  is, to all appearances, one of the factors in this inhibition. It is evidently not the only factor, however, since detoxification of the egg by ridding it of  $\text{CO}_2$  is not sufficient for development, but it is clear that intoxication by  $\text{CO}_2$  is one of the physiological causes of the unfertilizability of immature eggs. It is probably the same in the eggs of other anurans.

What is the *real role* of the spermatozoon in the metabolic changes that occur in the eggs of various species at fertilization? We have seen that the sperm is rich in oxidative enzymes and in hydrogen transporters. One might suppose that these enzymes would influence markedly the oxidations of the egg. The facts at hand in this connection do not favor this idea; on the contrary, they agree in picturing metabolic changes as a *consequence* of alterations in the egg during activation. The observations on the two extreme cases of the series that we have just reviewed, those of the sea urchin and of *Chaetopterus*, may be dealt with in terms of this idea. Warburg showed in his first studies (1908–1910) that artificial *parthenogenesis* in the sea urchin egg induced by hypertonic sea water, increases oxidation to the same degree as fertilization. This fact has been confirmed by Runnström. Furthermore, it is certain that it is not the enzymes in the sperm that stimulate the increase in gaseous exchange. It is interesting to recall that, according to Örström, activation of eggs of the sea urchin by hypertonic sea water, followed by treatment with relatively concentrated cyanide ( $M/250$ ), increases oxidation to the same extent as does fertilization. But cyanide in weak doses inhibits oxidation in the fertilized sea urchin egg, as we shall see later on. In *Chaetopterus*, activation is easily obtained by addition of isotonic KCl to sea water (F. R. Lillie). The eggs complete maturation and then exhibit a series of monaster cycles. Brachet has shown (1937, 1938) that the addition

of KCl entails a drop in  $O_2$  consumption that reaches a value of 50%, similar to that at fertilization. One may thus conclude that the respiratory changes that accompany fertilization, consisting of an increase or a decrease of these exchanges, are directly related to changes in the cytoplasm or in the cortex of the egg at this time. The spermatozoön plays only a secondary role in this regard and this conclusion is thus in perfect harmony with all that we know from experimental embryology.

In addition, another argument may be cited in support of this point of view. The eggs of the same female frog have a relatively constant respiration. When they are fertilized by the sperm of different males one would expect to see the rate of oxidation vary in the several lots if the amount of enzymes from the sperm has any effect. Brachet has found (1935) that this is not true, so the role of the sperm in the oxidations of the egg can only be a very slight one. Here, too, the result is in agreement with experimental embryology, since some of the observations of A. Brachet established that the rate of development is the same when eggs of the same female are fertilized by sperm of different males.

Nevertheless, it should be noted that in experimental *poly-spermy*, that is, the introduction of several sperm into the egg, there is an increase in respiration. Warburg (1908) noticed that the oxygen consumption of polyspermic eggs exceeded that of monospermic eggs by about 10%. This increase however, is too slight to attribute to the spermatozoön a decisive role in the regulation of the metabolism of the egg. In the frog, Brachet (1935) obtained a much greater increase (70%) in oxidation in polyspermic eggs, but this increase is only momentary and appears to be concerned with the growth of the numerous asters around the supernumerary sperm leading to an irregular segmentation (A. Brachet).

It is worth determining directly whether the metabolic variations in question are necessary for the normal course of fertilization. Fertilization, and even activation by appropriate chemical substances, remains possible when oxidation is prevented by addition of cyanide or monoiodoacetic acid. The fact that fertilization is rarely possible under anaerobic conditions stems from the fact that sperm movements rapidly cease in the absence of oxygen. Barron (1932) observed in *Nereis*, where the eggs and the sperm are both resistant to lack of air, fertilization is not prevented by complete anaerobiosis

when the gametes have been kept for 5 hours in a lack of oxygen. Development is very rapidly blocked, however, if the fertilized eggs are not returned to air. Activation by chemical substances remains effective in the presence of cyanide in the starfish egg (Brailley), egg of *Sabellaria* (Fauré-Fremiet), and the eggs of the mollusk, *Barnea* (Pasteels). The writer has observed that the activation of frog's eggs by chloroform takes place under anaerobic conditions or in the presence of KCN at a concentration where this poison inhibits the respiration about 90%.

Some work on the effects of monoiodoacetic acid on fertilization may now be cited. We know that this substance combines specifically with —SH groups (Rapkine, 1933; Dickens, 1933) and that because of this fact it inhibits glycolysis (Lundsgaard). Tyler and Schultz were the first to try this poison on eggs, and found that it does not prevent fertilization in *Urechis*. Similar results were found by Runnström (1933) in the case of sea urchin eggs, by Ellis (*Urechis*, sea urchin), R. S. Lillie (starfish), Pasteels (starfish, pholads). However, we have no assurance that monoiodoacetic acid really penetrates into the egg. Ellis has clearly shown that the amount of glutathione (reduced) of *fertilized* eggs decreases to one half in 95 minutes, but he has not proved that the monoiodoacetate is already combined in a few minutes with the —SH groups of the unfertilized egg. This lack of information caused Runnström to re-examine the question in 1935, using high doses ( $M/30$ ) and longer times (2–6 hours). He noted that the eggs submitted to such treatment remain fertilizable but that the larvae resulting show abnormalities. Thus, the penetration of the monoiodoacetate seems to be established. Consequently, Runnström concluded from these experiments that none of the chemical reactions blocked by monoiodoacetate (glycolysis, proteolysis by cathepsin) play a decisive role in fertilization.

In spite of appearances, we do not think that this conclusion can be accepted without reservations. We have noted (unpublished observations made in collaboration with L. Rapkine) that the respiration of unfertilized frog's eggs is not changed by monoiodoacetamide, a substance that acts like monoiodoacetic acid, but penetrates cells more easily. However, this amide rapidly inhibits the respiration of fertilized eggs and brings about cytolysis. It appears to do the same in the sea urchin egg, for Runnström has

observed that the respiration of fertilized eggs is lowered by monoiodoacetic acid. With regard to unfertilized eggs, it should be noted that they are decidedly less sensitive. Runnström admits that unfertilized eggs are less permeable or react more slowly with monoiodoacetic acid than fertilized eggs and we agree entirely with this opinion. It must be noted, finally, that, according to Rapkine (1936), the monoiodoacetic acid reacts much faster with glutathione than with proteins. If the latter play a role in fertilization, one might predict that monoiodoacetate will be effective, only after it has reacted with all the glutathione present in the egg. The results of Ellis, however, indicate that this condition is attained only after a considerable time. Thus, the question of the participation of the —SH groups bound to proteins in fertilization remains open and will be solved only when we have determined with certainty that these groups are effectively blocked under the experimental conditions employed by Runnström.

Before leaving respiratory metabolism, let us say a few words about the redox potential ( $rH_2$ ) of the unfertilized and fertilized egg. It is striking to find that this potential does not change following fertilization, even in the sea urchin egg, in which respiration increases so drastically at this time (J. and D. Needham, Rapkine and Wurmser, Chambers and collaborators). This is an interesting fact which shows that the  $rH_2$  is not a measure of the rate of oxidation. Thus, because a cell reduces a dye more easily it does not follow that its respiration is necessarily higher. The same reservation naturally also applies to cell organelles (mitochondria, vacuoles, etc.).

In general, fertilization does not change the pH of the egg (Vlès, Reiss, Rapkine and Wurmser, J. and D. Needham, Chambers), although in certain cases an acid forms at this time. It is probable that the buffering power of the egg is sufficient to maintain a constant pH. However, Gothié has reported an increase in alkalinity of the egg of *Ascaris* at fertilization and Trifonova an increase in acidity in the eggs of fish upon fertilization. Their methods, however, (vital staining) are not as reliable as those of their predecessors. Attempts to measure the pH of *Amphibian* eggs by means of micro-electrodes have given disparate results. Buytendyk and Woerdeman, for example, obtained a value of 8.5 for the pH of the fertilized egg as against 7.2 for the oöcyte, but Dorfman (1937, 1938) found only

an insignificant change (7.06 for unfertilized egg; 7.19 for fertilized egg). These divergent values are caused in part by the use of different electrodes, in part by the difference in pH at the two poles of the egg. For the unfertilized egg Dorfman found a pH of 7.0 at the animal pole and 6.5 at the vegetal pole. His technique appears to be definitely superior to that of the Dutch investigators, so that it would appear that fertilization does not bring about any large variations in pH in the frog's egg.

If one considers the metabolism of the *inorganic constituents* of the egg, particular interest is attached to *calcium*. This ion plays an important role in the beginning of maturation of the eggs of marine invertebrates (Dalcq, Heilbrunn, Pasteels). The research of Pasteels (1938) demonstrates that calcium stimulates the rupture of the germinal vesicle in a number of species. Many of the parthenogenetic agents lose their effectiveness when they are applied to eggs in calcium-free sea water: this is notably the case with fatty acids, ether, ultraviolet light, etc. These agents may simply cause an increased permeability of the egg to calcium, being merely "sensitizing agents," while calcium would be the real "activator." Certain substances such as alkalies and isotonic KCl stimulate maturation even in calcium-free sea water, but become ineffective if one eliminates the calcium present in the egg itself by treating it with citrate. Heilbrunn and Wilbur obtained somewhat similar results in *Nereis*.

Heilbrunn has suggested an interesting hypothesis to explain the role of calcium in physicochemical terms. He thinks that this ion is bound to the proteins localized in the cortex of the egg, and that at the time of activation one part of this complex is broken down and free calcium is liberated in the egg. All the activating agents would thus have one common effect, the liberation of the calcium from its combination in the cortex, resulting in a coagulation of the cytoplasm comparable to the coagulation of blood. In this manner, the 5 - to 6 - fold increase in cytoplasmic viscosity occurring after maturation in several species is explained.

Mazia, a student of Heilbrunn, undertook the verification of this hypothesis. He observed that the calcium content of the sea urchin egg was not changed at fertilization, but that the concentration of free, ultrafilterable calcium increases at the expense of combined calcium. The capacity of the egg cytoplasm to fix calcium

was thus decreased after fertilization. This fixation is probably a function of the acid groups of the proteins, the reduction in their number being coeval with the gelation of these proteins. These important conclusions have been confirmed in large measure by A. and M. Örström, who also found a liberation of calcium at fertilization, part of the egg content of this ion being eliminated into the sea water. The two valencies of the calcium ion are combined with the electronegative groups of the proteins. The total amount of calcium does not change for 15 hours following fertilization; then one finds an increase in this ion coincident with the formation of the skeleton of the larva. These authors assume that at fertilization there is an increase in the number of electronegative groups or a decrease in the pH of the intercellular fluids. There would then result a liberation of calcium, probably through the dissociation of a calcium proteinate. However, Öhman (1942) is of the opinion that calcium protects the eggs against cytolysis by lysocithin, this substance appearing to act especially on the lipids of the cortex.

Whatever may be the case, A. Monroy-Oddo has recently confirmed the fact that the sea urchin egg loses a large part of its bivalent cations after fertilization, this loss reaching a maximum of about 50%, for calcium and magnesium, one half hour after insemination.

The important role of calcium in the physiology of the egg is also accentuated by the observations of Moser, who has studied the fine granules located in the cortex of the eggs. These granules, seen earlier by Lindahl in 1932, are not displaced by centrifugation. They disappear at fertilization, their breakdown beginning at the point on the cortex where the sperm touches. They probably enter into the structure of the fertilization membrane, according to Runnström, Monné and Broman, and Runnström, Monné and Wicklund. This phenomenon, which is very rapid and precedes the elevation of the egg membrane, does not occur if one tries activation in citrated or oxalated sea water. The cortical reaction, then, needs calcium. It is obtained, however, in anaerobiosis (Kitching and Moser), which one could predict, since activation takes place in the sea urchin egg in the absence of oxygen. Moser believes that these results fall in line with the hypothesis of Heilbrunn of a liberation of calcium at the expense of a protein localized in the cortex.

It is interesting to compare these findings with those obtained by Mirsky on the *solubility of the proteins* of the egg of the sea

urchin. If the eggs are frozen at  $-77^{\circ}$  and then desiccated at  $-25^{\circ}$ , one obtains a fine powder that may be extracted at low temperature with  $M$  KCl, pH 7.3. Under these conditions, 82–85% of the proteins from the unfertilized egg dissolve; for the fertilized egg this becomes 69–72%. Part of the egg proteins, more than 10% of the total protein, thus becomes *insoluble* at the time of fertilization. The coagulation, which brings about insolubility, differs from denaturation by heat or by acids. In fact, it is not accompanied by a liberation of the —SH groups, being more like the related phenomenon of the reversible denaturation of myosin during muscular contraction (Mirsky). It is characterized chiefly by a loss in solubility, probably as a result of the destruction of the hydrated sheath that covers the protein molecules; the internal structure of these molecules, notably the —SS— and —SH bonds, would not be changed. The coagulation described by Mirsky begins three minutes after insemination, obtains its maximum in seven minutes, and is maintained thereafter unchanged for two hours. The mitoses of segmentation would thus not be associated with any change in the solubility of the proteins. In order to explain the increased resistance and elasticity of the egg after fertilization, Mirsky thinks that the elongated molecules associate at this time to form a network. In effect, the soluble proteins of the egg contain a fraction which is easily precipitated by ammonium sulfate; they have all the characteristics of fibrous proteins with elongated molecules: they are birefringent when oriented in parallel fashion by subjecting them to flow, and they exhibit a high viscosity. It appears reasonable that these fibrous proteins are precisely those which become insoluble after fertilization, anastomosing into a network and causing the increase in viscosity of the cortex of the egg at fertilization (Brown). This idea has the further advantage of integrating directly with the ideas of Frey-Wyssling on the molecular structure of cytoplasm.

Quite recently, Connors and Scheer have confirmed the existence of Mirsky's elongated protein and proved its homogeneity; in contrast to nucleohistone and myosin, respectively, Mirsky's protein contains neither desoxyribonucleic acid nor adenosinetriphosphatase.

These remarkable observations of Mirsky are connected with those of Mazia in the following way. At fertilization, the proteins become modified and simultaneously lose their bound calcium; we can even picture, according to the hypothesis, that it is the ends



of the elongate molecules, where calcium splits off, which unite to form the network. In summary, we may visualize the following process: at the time of fertilization a reversible denaturation of the proteins takes place with the liberation of calcium and the solution of the cortical granules. It may be noted here that numerous denaturing agents activate the unfertilized egg and that in the course of normal fertilization an acid is formed in the cytoplasm (Runnström: see later). The elongate proteins may then unite into a network possessing the consistency of a gel (coagulation of Heilbrunn and Mirsky). Such an interpretation of the facts, tempting though it may be, is, we repeat, hypothetical; we present it here only as a useful guide to further research. It will be seen at once that the explanations which take into account the submicroscopic structure of the cytoplasm (Frey-Wyssling) are more attractive than those which would give the chief role to oxidations. As a matter of fact, we will see on several occasions that the future of chemical embryology lies more in a study of the egg proteins than in the analysis of metabolism. The explanation just proposed, incomplete though it may be, has the advantage of bringing together a large number of apparently unrelated facts: the role of calcium in activation, the possibility of obtaining activation by the most varied physical and chemical agents, the changes in viscosity and elasticity that take place in the egg at fertilization, the importance of the cortical reaction. There is no doubt that the gaps that still exist will soon be filled; we have already attained a firm enough foundation to anticipate with confidence future, important progress in our knowledge of the rearrangements that the egg undergoes in the first phases of fertilization.

Meanwhile, let us turn to a review of the state of our actual knowledge of the metabolism of the egg best known to us in this respect, that of the sea urchin.

### 3. Chemical Changes Caused by Fertilization of the Sea

#### Urchin Egg

We already know that fertilization or activation by parthenogenetic agents produces a rapid and considerable increase in oxidation. The work that we will attempt to review briefly seeks to analyze this phenomenon. Consideration of this work takes us necessarily into a field which is more biochemical than embryological.

Let us first see if a relation exists between the low respiration

of the unfertilized egg and its particular stage of maturation. We know that the sea urchin egg can be fertilized only in the oötid stage, when it has completed its maturation. Lindahl and Holter thought of comparing the oxygen consumption of immature eggs (oöcytes in first division) with that of mature oötids and of fertilized eggs. In order to work under the most favorable experimental conditions possible, they used a small number of cells and made the measurements by means of the cartesian diver of Linderström-Lang. Thus, as is shown in Figure 24, the respiration of oötids

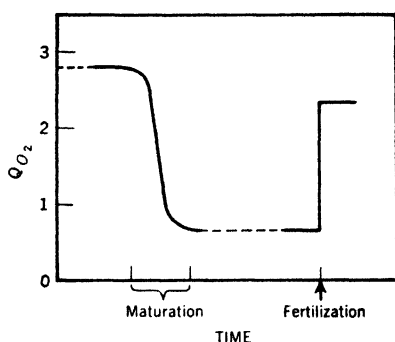


Fig. 24. Oxygen consumption during maturation and at fertilization in sea urchin egg (Lindahl and Holter).

is clearly lower than that of the primary oöcytes which still have the germinal vesicle. These latter have a somewhat higher metabolism than fertilized eggs.

These results are of considerable interest. They prove that, in accordance with the ideas of Whitaker, the metabolism of the unfertilized egg is very abnormal, and are also consistent with the theory of Bataillon, who considers the unfertilized egg to be a poisoned, asphyxiated cell. The observations of Lindahl and Holter help in understanding why such a marked increase in oxidation takes place during fertilization. If the primary oöcyte could be fertilized in this species the penetration of the sperm would bring about a small *decrease* in the gaseous exchange. What really distinguishes the sea urchin egg is the enormous decrease in respiration during maturation. It is here that we must look for the fundamental cause of the phenomenon discovered by Warburg. It is interesting to note in this regard that in the starfish egg the respiration of mature and immature oöcytes is of the same order of magnitude, according to Boell, Glancy, Chambers and Stern. In this species, however, fer-

tilization occurs shortly after the rupture of the germinal vesicle at the time when the first maturation spindle is at metaphase. The disintegration of the germinal vesicle is thus not sufficient to lower metabolism; the decrease in the latter, in the sea urchin, is dependent on a series of physical and chemical changes in the egg which take place gradually during maturation. Thus, Runnström (1923, 1924, 1928) noted a decrease in viscosity and permeability during maturation.

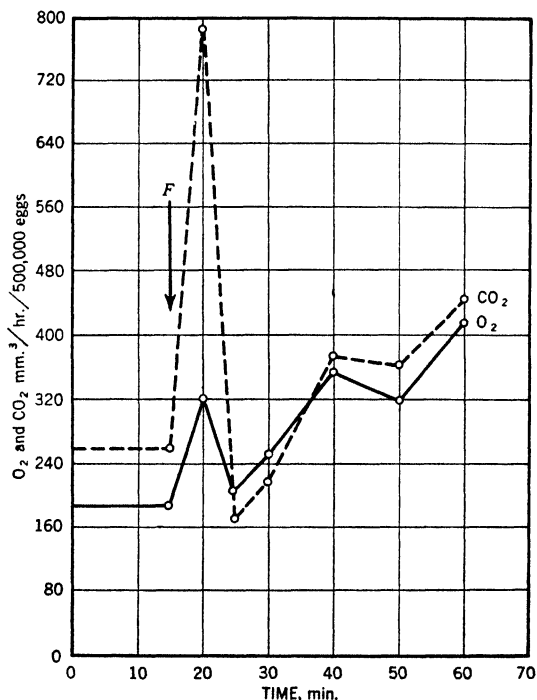


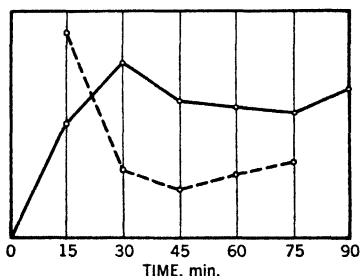
Fig. 25. Oxygen consumption and CO<sub>2</sub> production at the time of fertilization (↓) in the sea urchin (Laser and Rothschild).

tion; he also observed a change in the color of the cortex under dark field from white to yellow at this time. At fertilization the reverse changes take place, but more rapidly: viscosity and permeability increase and the cortex regains its white appearance. Maturation therefore entails, in the sea urchin, some profound modifications of structure and of metabolism. Fertilization brings the egg back to a condition resembling that of the primary oöcyte in several respects.

Whatever may be the causes of the inhibition of metabolism in the unfertilized sea urchin egg, fertilization quickly restores the oxidative level. Shearer has found that the increase in metabolism may be very striking; in fact it may begin a minute after insemination, at the time when the sperm have hardly reached the cortex; these claims, however, could not be confirmed by Runnström (1928, 1930). The most precise measurements are those made by Laser and Rothschild. According to these authors, there is a large but, temporary increase in respiration during the five minutes following fertilization. Respiration then falls to its initial value or even lower; this drop is also momentary and of such nature that at the end of 30 minutes the oxygen consumption attains the value usually found for fertilized eggs (Fig. 25).

Most investigators who have studied respiration of freshly fertilized egg agree that the second 15-minute period shows a

Fig. 26. Solid line = oxygen consumption of fertilized sea urchin egg; dotted line = the ratio of free  $\text{CO}_2/\text{O}_2$  (Runnström).



greater increase as compared with the first and third periods (Shearer, Runnström, 1933; Borei, 1933; Brock, Druckrey and Herkin). Figure 26 taken from Runnström's work illustrates this fact.

Concerning the *respiratory quotient*, we recall that it is very low for 5 or 10 minutes after fertilization; later it rises a little. Laser and Rothschild indicate in their work the values of 0.66 and 0.84 for the periods 5–20 minutes and 30 minutes after fertilization, respectively. Öhman (1940) obtained the figure 0.73 for the egg 1 to 2 hours after fertilization and deduced that oxidation occurred chiefly at the expense of the lipids at this time. These recent values are distinctly lower than those found in the early literature (Warburg, 1915; Shearer; Ephrussi; Runnström; Van Herk) which vary between 0.80 and 0.92. The causes of these differences must be found in the inherent errors of the older techniques that were used (see Öhman).

We will simply mention here the work dealing with *heat production* by the sea urchin egg during fertilization (Meyerhof; Shearer; Rogers and Cole). It has been discussed at length by Needham.

An important step in our knowledge was the discovery by Runnström (1930, 1932, 1933) of the *formation of an acid* at fertilization. R. Ashbel had previously found the production of an acid substance by unfertilized and fertilized eggs placed under anaerobic conditions, but it is without doubt that to Runnström belongs the credit for establishing the role of fertilization in this phenomenon. This acid production is brief and, in the presence of oxygen, can only be measured during the 15 minutes following insemination. The amount of acid formed in anaerobiosis is so much greater that it must be admitted that this substance is oxidized at the time when respiration increases. The most varied parthenogenetic agents will likewise bring about acid production. This fact is in harmony with Loeb's famous theory, according to which the spermatozoon exercises a cytolytic action on the egg. In fact, we know that cytolysis is accompanied by an acidification of the cytoplasm (J. and D. Needham, Runnström). The observations of Runnström have been extended by Borei to other species of sea urchins and the phenomenon has been analyzed recently by Laser and Rothschild. Acid production is very rapid and stops 5 minutes after fertilization; this becomes evident in figure 25 by the peak in  $\text{CO}_2$  production. The nature of the acid formed by the fertilized egg is as yet unknown. Runnström (1932) has shown that fluoride and monoiodoacetate do not inhibit its production; there is thus little probability that it appears as a result of glycolysis. The results obtained by Runnström after addition of hexosephosphates to the egg are contradictory (1933 and 1935). More recently, Rothschild has reported that phlorizin powerfully inhibits the acidification of a brei of sea urchin eggs, proving that the acid which forms under these conditions appears after a phosphorylation; but it is not demonstrated that the acid produced during cytolysis is the same as that formed after fertilization. We will return to this point later.

We owe to Runnström (1928–1935) a series of penetrating studies on the intimate mechanism of the increase in the oxidation at fertilization. Cyanide and carbon monoxide, which specifically block cytochrome oxidase, *do not inhibit* the respiration of the unfertilized egg; these poisons can even increase the respiration of the

egg (Örström 1935; Lindahl, 1938, 1939). On the contrary, cyanide and CO inhibit strongly the oxygen consumption of *fertilized* eggs. If one uses narcotics such as urethan which is adsorbed at the surface of the structures to which the respiratory enzymes are bound, we find that they interfere with the respiration of fertilized and unfertilized eggs to the same extent. Runnström (1928, 1930) has drawn the following conclusions from these data: In the unfertilized egg the cytochrome oxidase is not "saturated" by the oxidizable substrates, while in the fertilized egg it is. The granules which carry the respiratory enzyme are not in contact with the other components

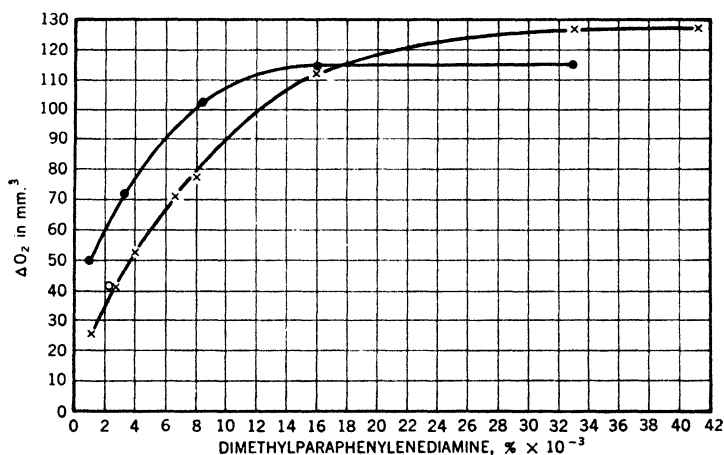


Fig. 27. Action of dimethylparaphenylenediamine on the respiration of unfertilized eggs (x) and fertilized eggs (●) of the sea urchin; the open circle corresponds to the oxygen consumption of fertilized eggs in the absence of dimethylparaphenylenediamine (Runnström).

of the chain of oxidations in the unfertilized egg; fertilization, by rearranging the intimate structure of the protoplasm, brings together the oxidase and its substrate. Runnström (1930) rejects the hypothesis that the increase in respiration is a result of the added activity of the dehydrogenases; in fact, he does not detect any difference in the rate of decoloration of methylene blue by unfertilized and fertilized eggs.

Runnström's theory, which claims that the respiratory enzyme of the unfertilized eggs is not saturated by its substrates, is confirmed in the work of Runnström (1932) and of Örström (1932). These

authors have added to eggs substances directly oxidizable by cytochrome oxidase without the need for dehydrogenases; these are hydroquinone and paraphenylenediamine. Figure 27 shows that these substances increase the respiration of unfertilized eggs considerably without having such a great effect on fertilized eggs. It is concluded that the unfertilized egg and the fertilized egg contain the same amount of cytochrome oxidase but that it does not function, because of lack of substrate, in the first case.

An interesting fact appears from the above: unfertilized eggs treated with these reagents are not activated. Thus, it does not suffice merely to raise the respiration in order to stimulate development.

In 1935 Runnström obtained some analogous results by adding to the eggs a substance capable of reversible oxidation and reduction, namely, pyocyanine. But he introduced at the same time some revisions of his interpretation. The amount of cytochrome oxidase and of dehydrogenases does not change at fertilization; the factor that stimulates an increase in oxidation at this time may be the formation of an intermediary transport compound which would act in the same manner as hydroquinone, paraphenylenediamine or pyocyanine. These carriers establish a bridge between the cytochrome oxidase and the dehydrogenases. The change in Runnström's concept brings him, in the final analysis, to the view that instead of a bringing together of the respiratory enzymes by a spatial rearrangement, he now considers that the contact is established by a carrier which is liberated at fertilization and unites the two systems chemically. By this revision of his ideas, Runnström attempts to integrate his theory with the present tendencies in biochemistry.

Certain investigators (Rubenstein and Gerard; Korr; Tyler and Humason) have tried to determine more exactly the role of oxidation in fertilized and unfertilized eggs by measuring the *temperature coefficients* ( $Q_{10}$ ). The results do not always agree, no doubt because of the technical difficulties. It seems clear, however, that the temperature coefficients of unfertilized and fertilized eggs are not identical, which would lead one to suspect that the reactions are qualitatively different. We will consider here chiefly the results of Korr. He concludes that the unfertilized egg respire through an iron-free, autoxidizable intermediary catalyst, while in the fertilized egg cytochrome oxidase, in which the active element is iron, is

functional. At fertilization there is established, conforming to Runnström's theory, a connection between the substrates of the dehydrogenases and the cytochrome oxidase by means of a carrier which is placed in circulation. Korr tends to think that this latter may be a cytochrome. Korr's views agree thus with Runnström in that he thinks the unfertilized egg is not using cytochrome oxidase, which is inactive and replaced by a different catalyst, free of iron and therefore insensitive to KCN.

But some recent investigations by Robbie lead to some very different conclusions. Re-examining the effect of cyanide on fertilized and unfertilized sea urchin eggs by an improved technique, Robbie has found, contrary to accepted ideas, that cyanide strongly inhibits oxygen consumption of *unfertilized* eggs. Maximum inhibition is about 55%, which is attained at a concentration of  $10^{-4}$  M cyanide; the effect of cyanide decreases with higher concentrations ( $10^{-3}$  and  $10^{-2}$  M), probably because of an abnormal reaction catalyzed by cyanide as Lindahl had earlier assumed. The inhibition of the respiration of fertilized eggs is however more marked since it attains a value of 80–85%. These new observations show that contrary to Korr's theory there are no profound qualitative differences between the metabolisms of unfertilized and fertilized eggs.

Likewise, Ballentine has arrived at a very different hypothesis from that of Korr and Runnström after a study of the *dehydrogenases* in the unfertilized and the fertilized egg. In a first paper (1938) he showed that the reduction of methylene blue in the fertilized eggs of *Arbacia* is from 93 to 233% more rapid than in the unfertilized eggs. His results are thus completely contradictory to those obtained on *Paracentrotus* by Runnström (1930) and Örström (1932), who deny that fertilization has any influence on the reducing power of entire eggs or breis. Let us add in passing that Ries (1937) observed a more rapid decoloration of methylene blue and Janus green under anaerobiosis after fertilization in the egg of *Paracentrotus*. Consequently, according to Ballentine, the dehydrogenases of the unfertilized sea urchin egg are much less active than those of the fertilized egg. The American author, using a new method by Quastel and Wheatley, has extended his research to other species (*Chaetopterus*, *Mactra*, oyster). In all cases he finds a parallelism between the activity of the dehydrogenases and respiration. Thus, in the egg of *Chaetopterus*, where respiration decreases



at fertilization, Ballentine observes a reduction in the activity of the dehydrogenases. In the sea urchin egg, on the contrary, their activity increases sharply. Finally, Ballentine showed that the addition of a carrier such as cresyl blue increases the respiration and the activity of the dehydrogenases in the unfertilized egg of the sea urchin, while this dye has no effect on the fertilized egg. Ballentine is of the opinion that this dye does not act as a intermediary in the chain of oxidations but that it has a direct effect on the dehydrogenases.

The nature of these dehydrogenases remains unknown. It is certain that the sea urchin egg contains coenzymes, but succinic dehydrogenase, triosephosphate dehydrogenase and glutamic dehydrogenase are lacking (Jandorf and Krah1).

The claim of Runnström that the amount of cytochrome oxidase does not change at fertilization has been contested by Ries. According to the latter, unfertilized eggs of the sea urchin give only a feeble indophenoxidase reaction (Nadi reaction), while fertilized eggs become intensely colored with blue. Ries deduces from this that a synthesis of this enzyme occurs during fertilization. We have already seen that the cytochemical method used by Ries is open to criticism and that its value as a quantitative measure is doubtful. It is surprising, too, that this striking phenomenon should have escaped other investigators who, like Ries, have applied the Nadi reaction to sea urchin eggs (van Herwerden; Sanchez y Sanchez; Schreiber). It must be noted that their results are sometimes strange. They maintain, for example, that in fertilized eggs the reaction is found especially in the vitelline membrane, which we hardly conceive as playing an important role in oxidation in the egg. Van Herwerden has reported that the intensity of the reaction increases during maturation, although we have just seen that respiration decreases at this time (Lindahl and Holter). During the later phases of the development of the fertilized egg, the intensity of the Nadi reaction decreases (van Herwerden; Ries; Ranzi and Falkenheim), but respiration continues to increase during this period. These few remarks reinforce the conviction that one must suspect the Nadi reaction as being influenced by too many contingent factors. The matter appears settled if one considers the observations of Krah1, Keltch, Neubeck and Clowes who quantitatively determined cytochrome oxidase in unfertilized and fertilized eggs. They definitely

concluded that the amount of this enzyme does not change at fertilization. Now these authors believe they are dealing with a true cytochrome oxidase, contrary to Ball and Meyerhof, who are inclined to assume the presence of a haemin closely related to this enzyme. This disagreement in detail is not of great importance and we can consider that this recent work confirms Runnström's opinion that *the amount of respiratory enzyme present in the egg does not change at fertilization.*

A number of investigators (J. Brachet, 1934; Lindahl, 1936; Ball and Meyerhof; Krahle, Keltch, and Clowes) have searched in vain for cytochrome in the sea urchin egg. Korr's interpretation, which identifies as cytochrome the hypothetical carrier that would appear at fertilization, can hardly be retained in face of this lack of evidence, and Korr himself has revised his opinion (1942).

Let us finish by an examination of some work dealing with the effect of fertilization on fragments separated by ultracentrifugation, according to the method of E. B. Harvey. Recalling that Shapiro found the greater respiration in the "heavy" fragment containing the pigment and the yolk, where the oxygen consumption was more than 88% of that of the entire egg, we see further that Ballentine (1939) reported that the heavy fragments contained, at equal volume, 70% more of the dehydrogenases than the intact egg. The parallelism between the figures of the two authors is an argument in support of Ballentine's thesis: that the intensity of respiration appears to depend on the activity of the dehydrogenases. Shapiro has examined, since 1935, the effects of the penetration of the spermatozoön on the metabolism of the two types of fragments. He found that the oxygen consumption of the light half increased by 270% just as did that of the entire egg at the same temperature. The respiration of the heavy fragment, on the contrary, is hardly affected by fertilization. Thus, the light fragment resembles the unfertilized egg, and the heavy half recalls the fertilized egg. Later research has bolstered this analogy. Shapiro (1940), for example, noted that in reality the respiration of the light half is cyanide-insensitive before fertilization, while KCN inhibits respiration after fertilization; the heavy fragments, unfertilized or fertilized, are cyanide-sensitive. In addition, paraphenylenediamine increases the oxygen consumption of the unfertilized light halves while it does not alter the respiration of the heavy fragments (Boell, Glancy,

Chambers, and Stern). It will be interesting to see how far the parallelism between the light fragment and the unfertilized egg on the one hand, and the heavy half and the fertilized egg on the other hand, can be extended. It is evident that the accumulation of granules in the anucleate fragment produces the same effect as fertilization. In light of this it may well be that the first hypothesis of Runnström contains a great deal of truth. Furthermore, it is clear that centrifugation, by displacing the granules, increases the activity of the dehydrogenases in the heavy fragment which in this manner is made to resemble the fertilized egg. It seems to us that it is not impossible that the rearrangements of cytoplasmic structure in the egg at fertilization result in an activation of the dehydrogenases.

It is undeniable that the intimate mechanism of the increase in oxidation at fertilization remains confused. Most investigators are in agreement on a number of facts, but there is one point of complete discord between the Swedish workers and Ballentine: whether there is a change in the activity of the dehydrogenases at fertilization. Ballentine's observations are certainly very impressive; the parallelism between reducing power and oxidation was found in the eggs of a number of forms where the respiration increases or decreases at fertilization. This parallelism also holds for fragments isolated by ultracentrifugation. The experiments of Örström are, on the contrary, relatively less numerous and it is to be noted that in three of them out of a total of five the reducing power of the fertilized egg was greater, by at least 30% over that of the unfertilized eggs. Thus it appears that one may accept Ballentine's conclusions, until proved contrary, with the hope that some new investigations will settle the question definitely. Certainly, it should be very interesting to follow the activity of the dehydrogenases during maturation. Among other questions, it may be asked if there is a progressive inhibition of these enzymes when the respiration decreases. Finally, we must hope that the future will bring some new data on the relation existing between the metabolism of the egg and the alterations of structure that it undergoes at fertilization; the experiments on centrifugation indicate a direct relationship such as Runnström first showed.

Up to the present we have paid little attention to the nature of the substances oxidized by the egg. In what follows, we will give in summary fashion what is known of the carbohydrate, lipid, and protein metabolism during fertilization in the egg of the sea urchin.

*(a) Metabolism of the Carbohydrates*

The presence of glycogen in the sea urchin egg and its utilization during development have been established by Ephrussi and Rapkine. More recently, Hutchens, Keltch, Krahl, and Clowes have determined that glycogenolysis is insignificant during segmentation, in spite of the intense mitotic activity; it is only between the 15th and 24th hour of development that it becomes considerable. It was first thought that glycogen would be metabolized as in muscle, that is to say, transformed into lactic acid, the latter then being oxidized aerobically. In effect, according to Perlzweig and Barron, the amount of lactic acid of the egg does rise slightly at fertilization, going from 2.7 to 3.2 mg per gram of protein. If one suppresses oxidation by cyanide, there results an accumulation of lactic acid just as in muscle.

These figures of Perlzweig and Barron make it probable that lactic acid and the unknown acid which Runnström found produced after fertilization are identical. Rapkine undertook the verification of the observations of the American workers. He reported (1931) that the amount of lactic acid in the egg decreases sharply about 10 minutes after fertilization, then rises and attains a maximum at the end of 50 minutes, that is to say, a little before the first division. Unfortunately, the method used is not free from criticism as Rapkine himself notes. This is why Runnström again took up the question in 1933. He concluded that there is not an accumulation of lactic acid at the time when the production of an acid detected by manometric methods is at a maximum, and this finding has been confirmed by Zielinski and by Örström and Lindberg, although van Herk found a slight increase. Thus, it seems correct to assume that the amount of lactic acid in the egg increases hardly at all at fertilization and that the acid described by Runnström is of a different nature. It does not appear likely that it is pyruvic acid, judging from the determinations of Örström and Lindberg and those of Barron and Goldinger; nevertheless one should not lose sight of the fact that, according to Barron and Goldinger, as well as Krahl, Jandorf, and Clowes, fertilized eggs oxidize pyruvate five times faster than unfertilized eggs. It also follows from the work of these authors that the metabolism of the sea urchin egg differs from that of muscle. In contrast to muscle (Szent-Györgyi, Krebs), the egg is unable to oxidize succinate and a keto-glutarate.

We owe to Zielinski the demonstration of a decrease in glycogen in the egg during the 10 minutes following fertilization. This fact, which has been confirmed by Örström and Lindberg, implies an active metabolism of the carbohydrates after the penetration of the spermatozoon; as shown in Figure 28 glycogenolysis diminishes sharply

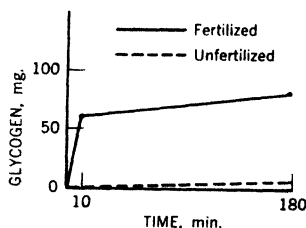


Fig. 28. Disappearance of glycogen in unfertilized and fertilized sea urchin eggs (Örström and Lindberg).

afterwards. More recently, Lindberg (1945) has determined that at the time of fertilization part of the free glycogen (lyoglycogen) becomes attached to proteins to become desmoglycogen.

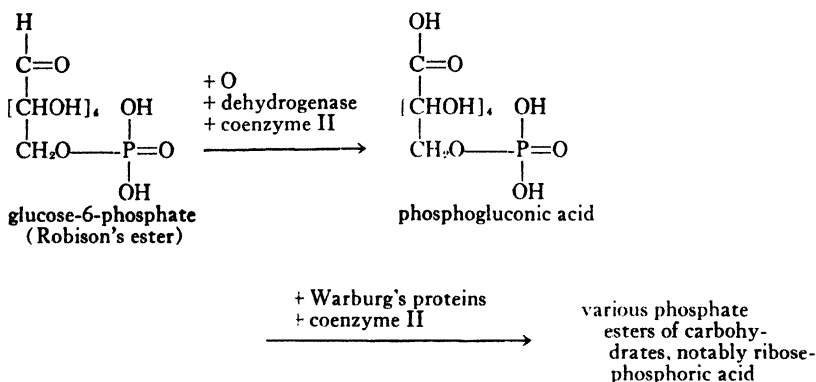
This decrease would be accompanied, contrary to Zielinski's opinion, by a decrease in the amount of reducing sugar (Örström and Lindberg). The quantity of glycogen and sugars disappearing corresponds exactly to the quantity of acid formed at fertilization. Örström and Lindberg thus affirm, as does Rothschild, that this acid originates from carbohydrate metabolism.

The fact that glycolysis is accompanied by pronounced changes in the distribution of the phosphate esters has not been ignored. However, there is unanimous agreement that the amounts of inorganic phosphates, of pyrophosphates, and of hexosephosphates are not modified at fertilization (Runnström, 1933; Zielinski; Örström and Lindberg).

Örström and Lindberg, in the course of a study on glycogenolysis in egg breis, established the fact that this process is accompanied neither by an accumulation of lactic acid, nor by changes in the phosphate esters; nor is it influenced by the addition of adenylic acid. An extract of cytolized sperm, however, accelerates it. It is blocked by phlorizin, a fact which leads one to believe that the glycogen is first phosphorylated (see Parnas, Cori in the case of muscle); the later utilization of glycogen leads to the formation of acid products following a pattern which is no doubt largely like that known for muscle.

Recent work of Lindberg gives a partial answer to the question of the utilization of glycogen. This author has discovered an unknown phosphate ester in the eggs which is also found in a number of

organs of vertebrates and invertebrates; it is probably a compound formed from phosphoric acid and propanediol and contains neither sugar nor purine. It constitutes 15% of the acid soluble P of the eggs. This substance accelerates glycogenolysis in sea urchin egg breis, and when Lindberg's ester is added to a brei together with a product of the oxidation of hexosephosphate, phosphogluconic acid, some acid soluble pentoses are formed. This ester probably favors the oxidative decarboxylation of hexosephosphate leading to the formation of ribosephosphoric acid (Dickens). It is noteworthy that the amount of acid-soluble pentoses increases slightly during normal fertilization and more markedly during cytolysis. These transformations may be formulated in the following way:



If we attempt to summarize the state of our knowledge of the carbohydrate metabolism of the sea urchin egg, we may say that fertilization stimulates the oxidation of carbohydrates, as evidenced by a diminution in the amount of glycogen. This glycogenolysis is not affected by monoiodacetate and fluoride, but it is inhibited by phlorizin, following a different pathway from muscle glycolysis. The acid which forms at fertilization appears in a quantity equivalent to the glycogen consumed at the time; the nature of this acid is unknown, but we can exclude lactic and pyruvic acids; it probably consists of a mixture of acids resulting from the oxidative decarboxylation of the carbohydrates.

#### (b) *Metabolism of the Lipids*

We know very little of these compounds. Observations by Hayes indicate that the amount of neutral fats of the egg decreases

during the first 9 hours of development. This author believes that fats are saponified to fatty acids and glycerol at fertilization, the glycerol being oxidized by the cyanide-sensitive respiratory system, while the fatty acids would correspond to the acid of Runnström, being oxidized by the cyanide-insensitive enzymes. This supposition is at present not supported by any experimental data. However, it will be recalled that, according to Öhman (1940), the fertilized egg has an R.Q. of 0.73, characteristic of the oxidation of lipids. More recently (1942), Öhman showed that the amount of phosphatides and of cholesterol in the egg decreased by 17% and 25%, respectively, in the course of 30–90 minutes following fertilization. Some changes in the amount of various lipids in the egg of *Arbacia* at the time of fertilization have also been observed by Monroy and Ruffo. These facts tend to indicate an intense metabolism of the lipids at this time without showing whether it occurs as soon as the spermatozöon contacts the egg.

*(c) Metabolism of the Proteins and Their Derivatives*

The suggestive work of Mirsky on the drop in solubility of the proteins after fertilization was reviewed earlier. Since this phenomenon is probably identified with the onset of denaturation, it would be well to see whether there is produced at fertilization a liberation of the —SH groups—a classical manifestation of the denaturation of the proteins. However, it will be recalled that, according to Mirsky, the amount of proteins with —SH groups does not vary during the coagulation which he discovered.

In general the determination of —SH groups by the nitroprusside reaction used on fixed or intact eggs is all that has been done. A positive reaction is interpreted as the presence of glutathione or of cysteine bound in the proteins. The importance of denaturation produced by reagents has unfortunately not received sufficient attention.

Shearer (1922) noted that the unfertilized eggs of the sea urchin gave a reaction of the —SH radical, the intensity of which varied from one batch to the other, but that the color became more intense a minute after insemination. Primary oöcytes, still immature, do not contain —SH groups. Shearer attributed the reaction exclusively to glutathione. Eggs failed to give the reaction with nitroprusside after they had been extracted with warm water, but the extract then gave a good reaction. Dulzetto, and subsequently Ries

(1937), have confirmed that the color increases in intensity after fertilization. They have, however, found that immature eggs also contain the  $\text{—SH}$  radical, particularly in the germinal vesicle. The contradictions between these authors and Shearer probably originate from the fact that they worked with eggs fixed in trichloroacetic acid and as a consequence the proteins became denatured; Shearer, on the other hand, placed living oöcytes in the reagent.

Rapkin (1931) has subsequently attacked the problem by controlling the cytochemical results by determinations of the glutathione. We shall have occasion to return to this work in connection with cell division and for the moment will consider only the following aspects of it. When one carries out the nitroprusside reaction in a test tube on a suspension of eggs treated with ammonium sulfate, a marked coloration is obtained with unfertilized eggs. The color in general tends to become more intense after fertilization, then the reaction decreases to the point where it is almost negative about a half hour after insemination. After this it reappears and obtains a maximum 20 minutes later. Rapkin found that the increase at fertilization in the amount of  $\text{—SH}$  groups in the egg is distinguishable only when the reaction is carried out under the microscope. It is so slight in the *in vitro* suspensions that the author concludes: If an increase in  $\text{—SH}$  groups really exists, the indication of it falls within the limit of error of the method. Titrations of glutathione by the iodometric method show that a decrease occurs for 30 minutes following fertilization. The method used as we now know was not absolutely specific and other reducing substances are included in the determinations. It is noteworthy that the amount of glutathione decreases even in the presence of cyanide which inhibits the oxidation of the  $\text{—SH}$  into  $\text{—SS—}$  groups. Rapkin deduced from this that this oxidation could take place in the absence of respiration.

Very recent work by Infantinella and La Grutta, who used a more reliable method for the estimation of glutathione, has shown that the concentration of the reduced form of the tripeptide increases nearly 4 times in the first 15 minutes after fertilization; the reduced glutathione then falls off progressively, but there is a slight peak just at the time of the first cleavage. Since the content of the eggs in oxidized glutathione remains constant and low, Rapkin's hypothesis of a transformation of the oxidized to the reduced form seems to be ruled out.

The interest in these observations is somewhat lessened by the

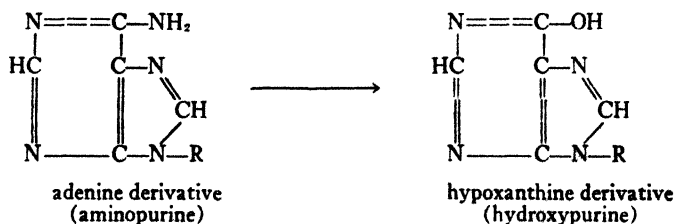


fact that, according to Ries, the eggs of various invertebrates behave very differently in regard to the above phenomena. In *Aplysia* the reaction is negative for unfertilized and fertilized eggs; in *Chaetopterus*, it is not modified appreciably by fertilization. If the latter always produced a denaturation of the proteins with the liberation of the —SH radicals, one would expect to find a constant increase in the reaction. Let us not lose sight of the fact, however, that the techniques leave much to be desired in regard to the amount of —SH groups in the egg proteins which are *not denatured*; we do not as yet have at our disposal a quantitative method for the determination of these radicals in the intact egg and this alone would answer the question. Thus the matter remains open at the present time.

There remain the very penetrating studies which Örström has devoted to the nitrogen metabolism of the sea urchin egg, especially the *ammonia production*. It is not our purpose here to present a detailed analysis; we will limit ourselves to the essential results, stressing the interesting discussion in which Örström attempts to integrate his conclusions into a unified concept of the fertilization of the sea urchin egg.

Örström states first of all that fertilization stimulates a marked, though temporary, *increase in the ammonia production*, this increased excretion of  $\text{NH}_3$  also being observed after activation by parthenogenetic agents. At the same time a substance that liberates ammonia by heat is eliminated (glutaminylpolypeptide?).

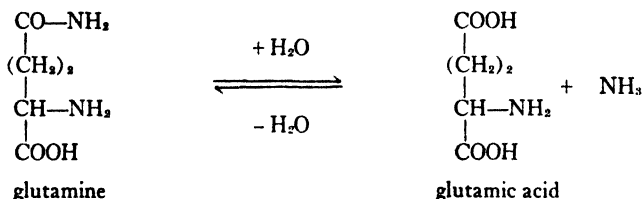
What is the origin of this ammonia liberated at fertilization? Numerous bits of evidence lead one to think that it comes from *aminopurines* (especially adenine), as is the case in muscle (Parnas), according to a reaction of the following type:



Örström, as a matter of fact, has found that unfertilized eggs reduced to a brei are able to deaminate adenosine and adenylic acid from muscle, the reaction being accelerated by the addition of cytolized

sperm. Furthermore, the amount of hydroxypurines in the egg, determined by a new technique, increases during the 10 minutes after fertilization. The aminopurine derivative which gives rise to the ammonia is not, therefore, adenylic acid as in muscle, but nucleic acid itself, in which the aminopurines undergo a deamination. This fact throws new light on the physiological role of nucleic acid to which we shall return later.

Another chemical reaction involving ammonia occurs during fertilization according to Örström. The fertilized egg is able to synthesize *glutamine* rapidly at the expense of glutamic acid and ammonia; the unfertilized egg is not able to accomplish this synthesis to the same degree. Nevertheless, sea urchin eggs, unfertilized or fertilized, can effect the reverse reaction, that is, the hydrolysis of glutamine into glutamic acid and ammonia. This is given in the following equation:



This reaction, reversible in the fertilized egg, goes only from left to right in the unfertilized egg.

If sea urchin eggs are placed in sea water containing ammonium salts, unfertilized eggs accumulate ammonia slowly, this accumulation being 10 to 15 times faster in fertilized eggs. Fertilization may thus modify the electro negative groups capable of combining with ammonia. We have seen that this is also true for calcium. We would thus have the reaction:



It is interesting to find that the addition of ammonium chloride brings about a very marked increase in oxygen consumption of unfertilized eggs, without affecting that of fertilized eggs. Higher concentrations tend to lower the R.Q. of the latter. Ammonium salts acting on unfertilized eggs increase nuclear volume: activation, however, does not go any further.

Örström has established that the sea urchin egg contains an

exceptionally high proportion of free amino acids (21% of the dry weight). Glycine is found in great abundance and no doubt takes part in the regulation of the pH of the egg. About 10 minutes after fertilization, one sees a sharp increase in the amount of nonprotein nitrogen soluble in trichloroacetic acid, resulting from the liberation of basic amino acids, histidine in particular. Thus during fertilization there is an important *proteolysis*, followed by a *resynthesis* of proteins. This phase of synthesis has also been noted in the egg of *Urechis* by Horowitz where the amount of nonprotein nitrogen goes from 46.8% to 40.7% of the total N between the 6th and the 30th hours of development. The nature of the enzymes that catalyze this proteolysis and final resynthesis is completely unknown; one immediately thinks of the peptidases, but Doyle has demonstrated that the amount of dipeptidase in the sea urchin egg remains unchanged at fertilization.

A résumé of the conclusions which Örström draws from his experiments will also give us the opportunity to recapitulate the essentials of our knowledge concerning the metabolism of the sea urchin egg at the time of fertilization. Figure 29, taken from Örström, indicates the various compounds expressed in millimoles as a function of time in minutes.

We see that the unfertilized egg behaves like a cell in a "latent" state, all the metabolic exchanges being slow. Fertilization begins with the attraction of the spermatozoa, stimulated by gamones (fertilization reaction of F. R. Lillie). This brief phase is followed by the "stimulation of development" (*Entwicklungserregung* of Loeb), which occurs for about 10 minutes after insemination. It is characterized from a chemical point of view by the splitting of large molecules, by the production of acids at the expense of glycogen, by the deamination of the aminopurines found in the nucleic acids, and by proteolysis. One must keep in mind that this first period of fertilization is reversible (Tyler and Schultz). If, for example one places eggs of *Urechis* fertilized 3 minutes in sea water slightly acidified, they behave at the end of 10 minute like unfertilized eggs and can be fertilized again if sperm is added to them. This experiment can be repeated several times and leads to polyspermic development.

Örström regards fertilization as stimulating three types of changes. It modifies the ammonia metabolism, as we have seen; it stimulates the breakdown of carbohydrates, and finally brings about

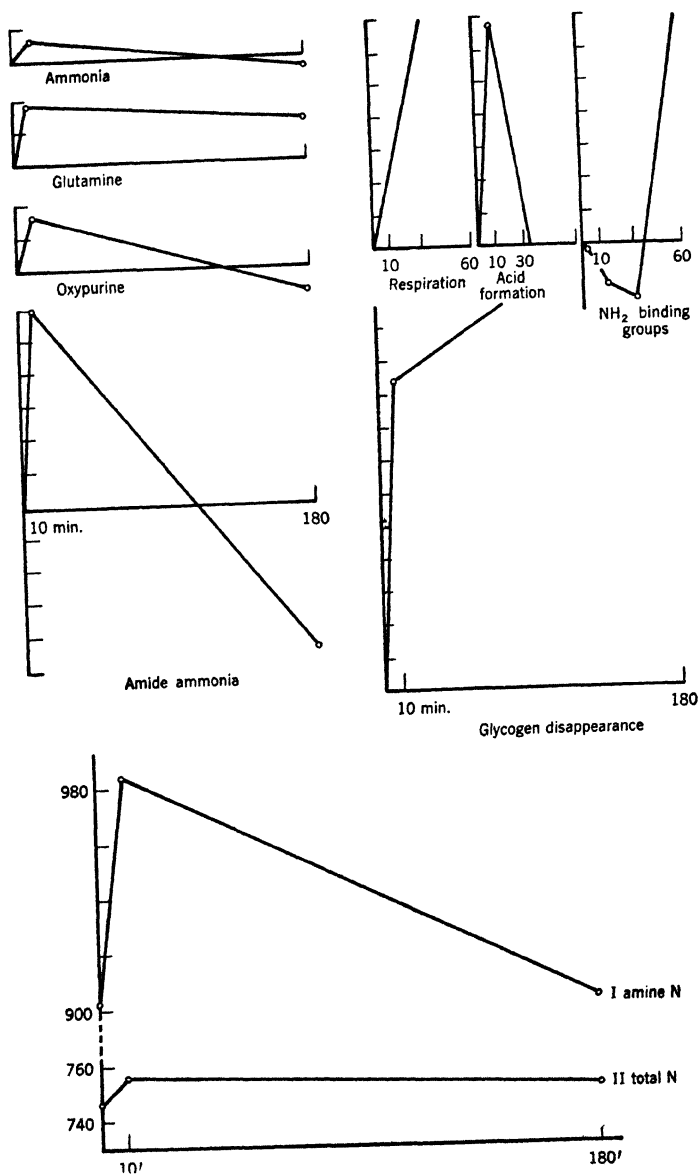


Fig. 29 Résumé of changes undergone by sea urchin egg at fertilization (Örström).



After the first 10 minutes, the substances formed during the first phase disappear, and the egg enters into a period of *resynthesis* which is made possible by the increased respiration. Örström ends with a comparison between the metabolism of muscle during contraction and that of the egg at fertilization. His points of similarity and difference between the two processes follow:

Muscular contraction	Fertilization of sea urchin egg
Production of $\text{NH}_3$ at expense of adenylic acid .....	Production of $\text{NH}_3$ at expense of polynucleotides
No changes in the amides .....	Synthesis of glutamine
Decrease in glycogen .....	Decrease in glycogen
Formation of lactic acid .....	Formation of acids differing from lactic acid
Formation of phosphate and changes in phosphate esters .....	Neither formation of phosphate nor changes in phosphate esters
Inhibition of glycolysis by monoiodacetate .....	Glycogenolysis not inhibited by monoiodacetate
Inhibition of glycolysis by fluoride ....	Glycogenolysis not inhibited by fluoride
Inhibition of glycogenolysis by phlorizin .....	Inhibition of glycogenolysis by phlorizin
Increase in respiration .....	Increase in respiration
Loss of solubility of proteins .....	Loss of solubility of proteins
Decrease in free $\text{K}^+$ .....	Liberation of free $\text{Ca}^{++}$

As we see, the work of Örström is an important contribution to our knowledge of the chemical basis of fertilization, his most striking conclusion being that which deals with *reversibility of the chemical reactions within the egg after fertilization*. Prior to his work the succession of hydrolyses and syntheses were completely unknown. The biological interest which attaches to them will soon be apparent.

One probably should make some reservations of secondary importance regarding certain of the conclusions of Örström. First of all, it is regrettable that Örström does not take into consideration the observations made in other laboratories. One thinks particularly of the work of Ballentine on the activation of the dehydrogenases at fertilization. Örström's interpretation, which views the increase in oxidation as a result of ammonia production, is not completely convincing, since he does recognize that the respiration of the unfertilized egg is not raised to a level characteristic of fertilized eggs by the addition of ammonium salts. It would be interesting to know if ammonium salts influence the activity of the dehydrogenases directly.

It seems that there is good reason to believe that the production of  $\text{NH}_3$  is not *exclusively* responsible for the increase of the metabolism and that other factors are superimposed.

To consider another point, it would seem that the order of the reactions proposed by Örström is somewhat arbitrary. In order to determine the succession of reactions, he relies on the fact that certain of them, carried out upon egg breis, are activated by cytolyzed sperm. The crux of the question is to know whether the results obtained on breis, where a dilution of the enzymes occurs, can be directly transposed to the intact egg, the effects of cytolyzed sperm not yet having been studied in all of the reactions taking place at the time of fertilization. Finally, one should recall that all of the changes reported by Örström are also shown by parthenogenetic eggs. The problem of determining the order of these reactions, some of which are probably coupled appears extremely difficult to resolve. One can not exclude the possibility that other chemical changes, not as yet known, take place at the same time.

These comments have only to do with details and they do not detract at all from the interest of the conclusions of Örström. On the contrary, one is amazed at the amount of progress accomplished by a limited number of investigators in the last 15 years. The only regret is that the analyses have been made primarily on the egg of sea urchin. One would like to know how far the facts thus acquired can be generalized and utilized for an over-all chemical theory of fertilization. The experimental work on oxidation showing a very particular rhythm in the egg of the sea urchin should make one rather cautious. Nevertheless, we believe that the time has come to integrate findings on the chemical level with the prevailing theories of fertilization.

#### 4. Biochemical Data and Theories of Fertilization

We will discuss very briefly the biological concepts that have arisen from studies on fertilization. A critical exposition of these hypotheses will be found in a book by Dalcq (1928) and in the later work of Dalcq, Pasteels, and J. Brachet.

##### *(a) Jacques Loeb's Theory*

Loeb was especially struck with the importance of the elevation of the fertilization membrane and with the necessity of two treatments to effect parthenogenesis (successive treatments with butyric

acid and hypertonic sea water), These facts led him to propose the following theory. The first period of fertilization or of parthenogenesis stimulates the elevation of the membrane by cytolysis of the cortex of the egg; simultaneously, oxidation becomes abnormal. The second period, which requires the presence of oxygen, is a sort of "corrective," the egg being rescued from the cytolysis which threatens to spread throughout it by the return to a normal respiration.

We can only touch on the biological criticisms raised against this theory. Dalcq has justifiably commented that the importance which Loeb attributed to membrane formation is certainly excessive. We also know now that parthenogenetic development can be obtained by the action of a single chemical substance in one application. This reservation is, nevertheless, not as serious as first appears, for one can conceive of a single parthenogenetic agent inducing the egg to pass through the two phases of cytolysis and return to normal, paralleling Loeb's assumption. We will reexamine this point apropos the theory of R. S. Lillie. Another objection has been made by F. R. Lillie to Loeb's theory on the basis that the sperm do not cytolize eggs except when sperm of a different species are used. But Lindahl (1938) has shown that sperm can cytolize the eggs of the same species under certain experimental conditions. If unfertilized eggs are treated with sodium sulfocyanide, for example, for 20 hours and then are fertilized, a cytolysis of the region in contact with the sperm is observed.

Upon considering the biochemical aspects of the discussion one notes that Loeb's method has been criticized on the basis that in the second treatment, the hypertonic sea water may be replaced by cyanide sea water (Bataillon), which should, according to all appearances, lower oxidation—contrary to Loeb's theory. But, as the experiments of Örström (1932) have shown, appearances are deceptive and the technique of Bataillon produces the normal increase in respiration. The fact is that the first visible change during the activation of the egg or during fertilization is a dissolving of the cortical granules (Lindahl 1932, Moser), beginning at the point of entrance of the sperm and preceding the elevation of the membrane, falls in perfectly with Loeb's theory of a cortical cytolysis; Lindahl (1938) calls attention to the fact that Loeb had postulated the presence of a "lysin" in the sperm. We now know that sperm con-



tain proteases and mucinases, the latter being so abundant in the testis that this organ is a source of their preparation. We also know that, according to Parat and Hibbard, the sperm of *Discoglossus* (Anuran) contains an enzyme, probably a mucinase, which digests the jelly from eggs. Parat thinks that the acrosome of the sperm contains a "proteolytic enzyme" and that its introduction into the egg results in parthenogenetic development. This enzyme would then be identical with the second factor in parthenogenesis in the eggs of anurans. Certainly it would be interesting to repeat the curious experiments of Parat and to pursue the study of the second factor on a chemical basis. The research of Bogucki, Einsele, and Rostand indicate that it is present in all nucleated cells and that it is destroyed at 55°. It is probably a protein with a small molecule distinct from thymonucleohistone.

Finally, the research of Örström unexpectedly confirms Loeb's theory in demonstrating that fertilization results in a hydrolysis of large molecules followed by a resynthesis. During the first phase, there is destruction of glycogen, proteins, and aminopurines combined with the nucleoproteins. All these changes are interpreted as an autolysis of the egg and are found during cytolysis. At the end of 10 minutes, the equilibrium of these reactions is modified and they shift toward synthesis. The parallelism between the chemical findings and Loeb's concept is quite perfect. One would like to know, however, how well the observations of Örström apply to other species.

Concerning oxidation specifically, it is clear that today much of Loeb's theory must be revised. The work of Whitaker established conclusively that the increase in oxidation is far from being a general fact. However, we must not forget that fertilization often results in quantitative and qualitative changes in respiration. Observations by Laser and Rothschild prove that the egg of the sea urchin has an abnormal R.Q. for several minutes following fertilization. Here again, however, one should know whether this phenomenon is general or whether it is peculiar to the sea urchin egg. Loeb concerned himself chiefly with the sea urchin egg, although he felt that his theory had general significance. In this special case, Loeb's theory does contain a good deal of truth and it is amazing to see the theory emerge almost intact after so many years. His

weakest point is unquestionably the role attributed to oxidation. This appears to be chiefly a contingent factor, since certain eggs are able to be fertilized under anaerobic conditions.

(b) *R. S. Lillie's Theory*

Ralph Lillie studied the starfish egg, the biochemistry of which, especially, is very imperfectly known. In contrast to Loeb, he believes in a single factor hypothesis, activation consisting in a progressive reaction that can be interrupted or stimulated at will. *Asterias* eggs can be activated either by acids or by heating, but if one stops the action of acids by replacing the eggs in normal sea water, activation is arrested; it begins again if they are replaced in acid medium or treated with heat.

R. S. Lillie attempted in 1931 to put his theory on a chemical basis. According to this, activation would be caused by the formation of an "activating substance," appearing as a result of reactions between certain specific elements in the cytoplasm. This reaction would occur only at a definite pH, which explains the role of acids. Activation would be complete when a certain concentration of the active substance had been obtained; failing this, one would obtain only a "partial" activation. Now, the egg does not respond as well to activating agents after the formation of the first polar body, since one of the precursors of the "activating substance" is probably destroyed at this time. Activation by acids or by heat is facilitated if one adds cyanide or if the eggs are placed under anaerobic conditions. Thus, one of the precursors of the "activating substance" is oxidized by the egg. Cyanide could also favor the formation of an acid such as lactic acid, which is produced in sub-threshold concentration and thus does not give complete activation.

R. S. Lillie arrives finally at the following concept. Activation is the end result of a number of physicochemical phenomena beginning with the hydrolysis of a substance localized in the cortex of the egg, this hydrolysis giving rise to the "activating substance" that stimulates development when its concentration passes a certain critical value. The action of acids consists in the liberation, by acidification of the protoplasm, of a precursor of the "activating substance"; heat acts similarly by producing an acid in the cytoplasm. The author is of the opinion, without supporting proof, however, that the hydrolysis concerns a phosphagen such as in mus-

cle. The hydrolytic reaction thus involves the splitting of arginine phosphate or creatine phosphate into arginine (or creatine) and phosphoric acid.

More recently, Lillie (1941) has brought new experimental evidence in favor of the idea that the activation reaction in *Asterias* eggs is a single process and that it takes place mainly in the cortex.

Runnström (1932) has discussed R. S. Lillie's ideas and has attempted to correlate them with his own observations on the egg of sea urchin. He points out that anaerobiosis effectively increases acid production during fertilization, and that these acids disappear by oxidation, as Lillie assumed. He notes also that all the parthenogenetic agents tried do stimulate this liberation of acid. The fact that the amount of free phosphate in the egg does not change during fertilization is contrary to Lillie's theory of phosphagen splitting. Runnström correctly emphasizes the hypothetical character of the "activating substance." For him, activation results mostly from a coupling between respiration and the disappearance of the acid substance as the result of a resynthesis analogous with that occurring in muscle. It is known that in the latter, four-fifths of the lactic acid formed is resynthesized into glycogen at the expense of the energy furnished by the oxidation of the remaining one-fifth (Meyerhof). If this coupling is absent in the egg, the acid will continue to accumulate and finally stimulate cytolysis. It can be seen that the coupling—easily attained before maturation—may be more difficult to effect later. Thus we can explain the fact that activation is only partial when it is effected after the formation of the first polar body.

Örström (1941) has proposed another interpretation. He supposes that the first hydrolytic reaction which gives rise to the "activating substance" in the cortex would be the production of ammonia at the expense of aminopurines. He does not, however, support this idea with direct arguments and it is well to remember that dilute ammonia (0.01 *N*) is a good activating agent in *Urechis* (Hiraiwa and Kawamura, Tyler and Bauer); activation by ammonia in this species has the same effect on oxidation as does fertilization (Tyler and Horowitz). One should recall, however, that in the egg of the sea urchin Örström has obtained merely a swelling of the nucleus after treatment with ammonia.

Lillie's theory, like that of Loeb, thus rests on certain chemical bases. The question of whether activation is accomplished in one or

two steps can not as yet be answered clearly. Lacking fundamental experiments one can not adopt a decisive position. It is necessary to know, for example, what chemical changes stimulate the first and second steps, respectively, in Loeb's method. This important gap could easily be bridged now that we know more about the chemistry of the sea urchin egg, thanks to the work of Runnström, Örström, Ballentine, Mirsky, etc. It will really be very interesting to see if the first step (butyric acid treatment) produces simply hydrolyses and the second step (hypertonic sea water) is necessary for resynthesis. One can offer in support of this possibility an observation by Örström. If eggs are activated by hypertonic sea water, the ammonia production follows a curve analogous to that of fertilized eggs, although the maximum is attained somewhat later; but, while ammonia disappears in fertilized eggs, it does not generally behave the same in activated eggs, which, in addition, cytolize rapidly. It is evident that if the treatment of eggs with butyric acid causes the ammonia produced to disappear, an essential argument in favor of Loeb's theory is available.

Again we must raise the question whether the metabolism of starfish and sea urchin eggs is perhaps different. It may be asked if treatment by heat or acids stimulates hydrolyses followed by syntheses in the starfish egg as fertilization does in the egg of the sea urchin or whether the reactions are radically different in the two species? Perhaps it is a mistake to think of the theories of Loeb and of Lillie as antagonistic. It is not impossible that each is valid for the particular species on which it was worked out (sea urchin or starfish). It would be pointless to try to include different types of eggs in the same theory if this were true. We see once more the necessity of extending the knowledge acquired from a study of the sea urchin egg to other species, that of the starfish in particular; it will only be when we have at our disposal exact information on this subject that it will be possible to find an answer to the many questions which arise at present.

#### (c) *F. R. Lillie's Theory*

We have already seen that this investigator attributes a major role to fertilizin: the attraction of the spermatozoön to the egg and the fusion of the two gametes. The ideas of F. R. Lillie have been vigorously criticized by Dalcq (1928, 1936) with regard to a

general theory of fertilization. The term fertilizin appears misleading to him and he prefers ovulin to it.

At the present time, fertilizin appears to be a mixture of gamones. The existence of these substances, we have seen, is no longer in doubt in a variety of species, ranging from the lamprey to the sea urchin; the chemical nature of the gamones remains debatable, but the problem is in a good state of progress. These gamones certainly play an important part in "external fertilization" (F. R. Lillie), attracting the spermatozoön toward the egg and permitting it to penetrate the surrounding membranes, but their intervention in the fusion of gametes is more debatable. These substances, by agglutinating the spermatozoa of other species, constitute a protection against hybridization. However, the gamones are only aids, although important ones, in fertilization, the latter often being successful in eggs that are free of jelly. The term gamone is certainly preferable to that of fertilizin. It indicates the origin of these substances, which are formed by the gametes somewhat like hormones; it also expresses the idea that we are dealing with chemical substances which bring about or favor the union of the two gametes.

The question of gamones has a real interest. Most of the criticisms of Dalcq are, nevertheless, pertinent, for one can not explain all of the phenomena of fertilization on the basis of these substances.

#### *(d) Bataillon's Theory*

Like Loeb, Bataillon concerned himself with the elevation of the membrane. He saw in this a reaction that leads to an excretion of substance from the unfertilized egg, intoxicated because it is inhibited by the products of its metabolism. (The extrusion of the vitelline fluid, and the increase in permeability during fertilization assure the elimination of the toxic products and permit development to proceed.)

The objection made by Dalcq and Pasteels that the elevation of the membrane is lacking in the eggs of certain species such as the pholads is not decisive. One can visualize without difficulty the elimination of wastes without the visible elevation of a membrane. Bataillon regarded the increased excretion of  $\text{CO}_2$  during fertilization in the frog egg (Bialaszewicz and Bledowski) and the egg of sea urchin (Vles) as an indication of detoxification. Actually, however, two very different phenomena are involved. In the frog egg

there is a retention of  $\text{CO}_2$  while the egg is in the genital tract and there are reasons to believe that in this case the  $\text{CO}_2$  contributes to the narcotization of the egg (Bataillon; Dalcq, Pasteels and J. Brachet); in the sea urchin egg the "burst of  $\text{CO}_2$ " results from the production of acids which react with the bicarbonates of sea water. It is debatable whether liberation of acids and elimination of  $\text{NH}_3$  indicate detoxification. Örström (1941), without alluding to the ideas of Bataillon, placed a series of modifications which accompany fertilization under the heading "detoxification" (*Entthemmung*); these included hydrolysis of the proteins, colloidal changes, coagulation of the proteins. For example, Örström thinks that the enzymes are bound in inactive complexes before fertilization, stimulation liberating them and rendering them active. This explanation differs from that of Bataillon, but the final result remains essentially the same.

In the same way the conclusions of Whitaker on the metabolism of the egg at the time of fertilization can be made to conform with Bataillon's theory. The unfertilized egg appears to Whitaker as a cell with an abnormal metabolism, fixed in a state resembling anaesthesia. Fertilization brings it out of this narcosis and regulates oxidation. The particular case of the sea urchin egg seems to be most suggestive. According to the observations of Lindahl and Holter, oxidation becomes progressively lower during maturation, thus exhibiting in a concrete way the progressive anaesthesia of the egg. (At this time there is probably an inhibition of certain respiratory enzymes, such as the dehydrogenases; fertilization lifts this block suddenly, the dehydrogenases recover their activity, and oxidation shoots upward).

Finally, recalling the old observations of Bachmann and Runnström demonstrating that the freezing point of the fertilized egg was much higher than that of the unfertilized egg, we must conclude that the egg loses osmotically active substances at this time. This deduction was verified experimentally by Krogh, Schmidt-Nielsen, and Zeuthen, who further showed that the phenomenon was not as marked as Bachmann and Runnström claimed. All these facts are in perfect accord with the ideas of Bataillon on the mechanism of the formation of the perivitelline membrane and the perivitelline fluid. They are also in agreement with the observations of Fauré-Fremiet, who showed that the membrane in the egg of *Ascaris* is built up at the expense of glycogen liberated at fertilization. In-

cidentally, glycogen may be eliminated during fertilization in the frog egg and may pass into the perivitelline fluid (Konopacki and Konopacka). These observations are, however, purely cytochemical and one can ask whether the Polish authors were not victims of artefacts, since the difficulty of fixing glycogen *in situ* in amphibian eggs is well known (Pasteels).

It is seen that arguments on a chemical level favoring Bataillon's ideas are not lacking. In a number of cases, the unfertilized egg appears as a poisoned cell and fertilization brings it out of its inertia by a purifying reaction. The case of eggs that do not show elevation of the membrane remains, nevertheless, somewhat embarrassing. It should be especially valuable to study the chemistry of the eggs of pholads and to see if one can not detect in them some sign of the elimination of wastes; these eggs are fertilized in the stage of the primary oöcyte, which still possesses its germinal vesicle. It is during maturation, however, that inhibition occurs in the sea urchin egg. It is possible, then, that in the egg of the pholad the poisoning is less profound than in that of the sea urchin and that the elimination is not accompanied by a membrane reaction visible under the microscope. It would be useful to determine whether it is the mixing of the nuclear sap and the cytoplasm that brings about the inhibition of the egg. This question has not been examined with all the attention which it merits. Whitaker said that the rupture of the nuclear membrane has hardly any effect on oxidation in the egg of *Nereis*, but perhaps the passing of the nuclear sap into the cytoplasm has a slow, gradual effect. This whole field, so interesting both from the point of view of embryology and of cellular physiology, remains to be explored.

One last point remains before leaving the subject. If the elimination of toxic substances is a necessary condition of fertilization, it is not sufficient to insure development. It is evident that one does not achieve parthenogenesis in the frog egg simply by freeing the eggs of their CO<sub>2</sub>. Dalcq, Pasteels, and Brachet, as well as Tyler (1937), have noted that some eggs can segment without membrane elevation. The elimination reaction such as conceived by Bataillon is thus not the only factor upon which development depends.

(e) *Dalcq's and Heilbrunn's Theories*

These authors have emphasized the role of calcium. Dalcq (1928) considered that the immediate cause of the activation ap-

peared to be a rearrangement and a redistribution of the salts. (This change could be only a subtle one, since the permeability of the egg to various ions does not necessarily change in the same way during fertilization.) These ions would then react with the colloids of the egg cytoplasm. We have seen above the view maintained by Pasteels, who attributes to calcium the role of "réalisateur" during the rupture of the germinal vesicle; other activating agents would only be "sensibilisateurs" or adjuvants.

(This concept resembles that of Heilbrunn, who assigns to calcium a major role in all of the phenomena of partial coagulation of the cytoplasm. It controls the increase in viscosity and the gelation which characterizes fertilization. Dalcq (1928) has criticized Heilbrunn for mistaking cause for effect, but this criticism seems to be extreme. It is more and more apparent that the rearrangements of the colloids, notably their reversible denaturation, play a prominent role in a number of physiological phenomena, such as fertilization, mitosis, muscular contraction, etc. It is certain that the observations of Mazia, of A. and M. Örström and of Monroy-Oddo, on the liberation of calcium after insemination, and those of Mirsky on the decrease in solubility of the proteins at this time, add to Heilbrunn's theory the biochemical support which it lacked. It evidently remains to be proved that the two phenomena are linked, but this eventuality now seems very probable.

Lindhahl (1938) criticized the theory of Heilbrunn on the following basis. After having washed eggs five times in calcium-free sea water and having placed them in a mixture of sulfocyanide and calcium-free sea water, he obtained the beginning of activation. But, according to A. and M. Örström, these washings do not suffice to rid the egg completely of its calcium. Treatment with calcium-free sea water causes a diffusion of calcium out of the egg. The experiment of Lindahl merely shows that activation is still possible after one decreases the calcium content of the egg; it does not prove that this phenomenon is not accompanied by a liberation of calcium at the expense of some complex in the egg.

## 5. Conclusions

At the end of this somewhat diffuse chapter let us try to summarize in a few words the more significant phenomena of fertilization. The unfertilized egg appears to be a cell which falls slowly



into a state of inhibition which becomes more and more severe. Its permeability decreases, and its oxidation changes to an abnormal condition, both qualitatively and quantitatively. It is surrounded by a jelly containing the gynogamones; these complex proteins attract the spermatozoa, activating their movements and tending to agglutinate them. They are elaborated by the egg when it reaches its maturity, perhaps originating from a cortical reaction (Dalcq, Pasteels, and Brachet). The spermatozoa have a high metabolism by virtue of their wealth of respiratory enzymes; they produce androgamones, which partially neutralize the gynogamones and ensure the exact mechanism of the fusion of the gametes. Among these androgamones is found a lysin of a protein nature, which permits the sperm to traverse the mucous jelly and reach the egg. Recent experiments by Ruffo and Monroy in Italy suggest strongly that this lysin is identical or very similar to hyaluronidase.

When the latter is in contact with the sperm, a series of important changes, the chronology of which is not exactly known, occurs. Permeability increases and the elimination reaction of Bataillon takes place, characterized by the excretion of osmotically active substances; at the same time the cortex undergoes transformations: certain granules are seen to disappear. Often, respiration is modified, and sometimes the R.Q. changes, indicating a qualitative alteration of the metabolism. In the sea urchin egg, the only case well known at present, a series of hydrolytic splittings is detectable, as well as a liberation of ammonia at the expense of the aminopurines combined with nucleic acids, glycogenolysis with the production of derived acids, and proteolysis, all reactions which recall to an amazing extent the cytolysis theory of Loeb. The liberation of calcium also occurs in this first phase, perhaps accompanying the cortical reaction and the breakdown of the granules of Lindahl and of Moser. In the second phase, which may correspond to the second period of Loeb, a partial resynthesis of the substances that had been hydrolyzed takes place. In the course of the first 10 minutes, the structure of the egg proteins changes; their solubility decreases, their elongate molecules anastomose to form a network; they probably undergo a reversible physiological denaturation. It is well to recall at this point that activating agents are all able to denature proteins (heat, acids, concentrated salts, ultra-violet, etc.).

We see that this brief résumé faces us with some fundamental ideas with which the experimental embryologist is already familiar: the roles of fertilizin, of the initial cytolysis (Loeb), of the hydrolyses and the acids (R. S. Lillie), and finally of calcium (Dalcq, Pasteels, Heilbrunn). Now that chemical embryology can pursue its analysis in detail, we may more than ever expect in the future the fusion into a coherent whole of the various embryological theories which have successively thrown more and more light on the troublesome problem of fertilization.



## *CHAPTER V*

# **Cleavage: the Relation of Metabolism to Cell Division**

We know that fertilization of the egg is followed by its division into smaller and smaller cells. This stage of development is characterized particularly by the number and rapid rate of cell divisions; it leads to the formation of the morula and then the blastula, but morphogenesis proper does not as yet begin. We know, however, that the egg undergoes alterations; cell movements begin which will insure the proper spatial distribution of cells during the course of gastrulation. From the biochemical point of view, the study of cleavage becomes reduced to analysis of mitotic division with all the consequences that are involved.

The morphology of mitosis is too well known to review here. The phenomenon of most importance is undoubtedly the division of the chromosomes into two halves that are precisely identical, qualitatively and quantitatively. It is this division that makes for the equal distribution of the chromatin and the genes into the daughter cells. This reduplication of chromosomes leads to a synthesis of chromatin that one can follow chemically by determinations of the thymonucleic acid content of the egg in various stages of development. We will reserve this aspect of the problem for the next chapter. We will limit ourselves here to a study of the biochemical changes that occur in cells during mitosis and we will attempt to correlate the results thus obtained with cytological changes.

### **1. Respiratory Metabolism During Cleavage**

It is a fairly general fact that oxygen consumption increases slowly during cleavage; this increase occurs regardless of the variations in the rate of respiration brought about by fertilization. We

know, for example, that the blastula has a higher rate of oxidation than the fertilized egg, in the case of the sea urchin (Warburg, Gray, Lindahl, 1936, 1939; Lindahl and Öhman, 1936, 1938: Fig. 30) as well as in that of *Chaetopterus* (Whitaker, J. Brachet). The same increase is exhibited in amphibian eggs where the oxygen consumption is not changed by fertilization, as we have already shown (1934). Certain investigators have nevertheless denied the existence of an increase in oxidation during cleavage in Amphibians (Parnas and Krasinska, Stefanelli), but they did not attempt to measure respiration continuously during this period. Furthermore, examination of the data of Stefanelli shows a rise of from 20 to 50% in oxygen consumption, an increase similar to that which we have observed and which was confirmed by Atlas and by Wills:

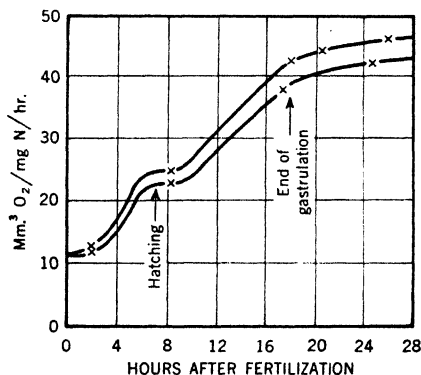


Fig. 30. Oxygen consumption of sea urchin egg between hatching and gastrulation (Lindahl).

All investigators who have considered the question agree with Warburg in recognizing that the increase in oxygen consumption does not bear a direct relation to the number of cells; for, indeed, respiration increases much more slowly than the number of nuclei. This fact is discussed at length by Needham in his book (1931) and recent research has only confirmed this relationship.

Are we justified then in postulating a relationship between the division of cells and increased metabolism in the course of cleavage? Some investigators are inclined to deny any relationship because the respiration of unfertilized eggs also increases with time and naturally these eggs are not undergoing cleavage. However, the recent publications of Tyler, Ricci, and Horowitz have shown that the increase in the gaseous exchange of the unfertilized egg is an artefact. In-

deed, it does not occur at all if necessary precautions are taken to insure aseptic conditions; hence, it is caused by proliferation of bacteria during the period of measurement, a proliferation which the cytolysis of some eggs in the manometric vessels increases. We have ourselves never obtained an increase in oxygen consumption of unfertilized eggs of the frog during periods ranging from 15 to 20 hours; these eggs are protected against cytolysis and bacterial contamination by their mucous jelly. During this same interval the respiration of fertilized eggs increases from 9 to 15 mm<sup>3</sup> of O<sub>2</sub> per 100 eggs per hour.

There is, however, a case in which the possibility of a relation between mitosis and oxidation is suggested. It is the case of polyspermy which increases metabolism, as we have already seen in the preceding chapter. This increase, slight in the sea urchin (Warburg), is very marked in the frog's egg where we have found a respiratory increment up to 70%. It is probably significant that this rise is only temporary and coincides with the appearance of voluminous asters which radiate out from the supernumerary spermatozoa.

The measurement of oxygen consumption has been extended to fertilized eggs in which cleavage has been inhibited by the action of various narcotics such as phenylurethan. Warburg pointed out as far back as 1910 that this substance stopped cell division in the sea urchin egg, primarily in the formation of the cleavage furrow. In spite of this, however, there were no appreciable changes in respiration. Runnström (1928) has given this question great attention and found that narcotics of the urethan series inhibit cytoplasmic division without affecting nuclear division, with the result that many nuclei form in the undivided cytoplasm. Under these conditions narcotics strongly inhibit respiration, which, however, tends to increase as segmentation progresses, just as in the controls. Nuclear division is possible when the oxygen consumption is reduced to 35% of the normal. Finally, Tyler and Horowitz have studied the effects of phenylurethan on the fertilized eggs of *Urechis* and, as indicated in Figure 31 (curves A and D), the narcotic exerts its effect only when respiration begins to increase. This increase is much less rapid in the treated eggs than in the controls and these investigators attribute the rise to the maintenance of nuclear divisions, while the divergence between the two curves is the result of suppression of cytoplasmic divisions in the narcotized eggs. In the case of the frog's egg, where

we have found (1934) that phenylurethan inhibits cytoplasmic division without affecting the nuclei, this substance markedly lowers oxidation.

Recently, Fisher, and Henry and Fisher, have reexamined the case of the sea urchin. They conclude from their studies that the fertilized eggs contain two distinct respiratory systems, of which only one is sensitive to ethylurethan. Mitosis is stopped in the presence of this narcotic when this system is completely inhibited. The fraction of the respiration sensitive to ethylurethan is lacking in the unfertilized egg and it is this that furnishes the energy necessary for mitosis. The conclusions reached by Fisher and Henry have

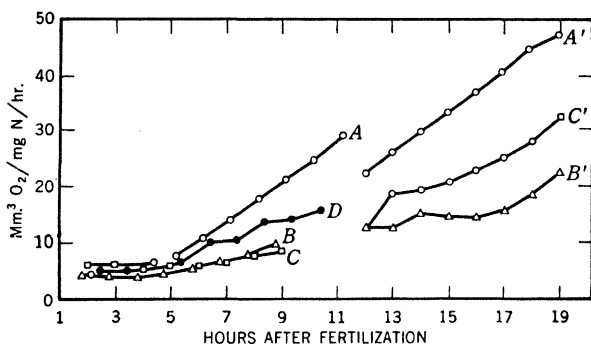


Fig. 31. Rates of oxygen consumption in the egg of *Urechis*: A, A', fertilized eggs; B, B', activated eggs differentiating without cleavage; C, C', parthenogenetic eggs; D, eggs treated with phenylurethane (Tyler and Horowitz).

been extended by Moog to the frog egg, and here too there appears to be a special respiratory system sensitive to narcotics and necessary for mitosis.

The use of narcotics entails certain difficulties. Indeed, there is no proof that these substances specifically influence cell division and that their introduction into the egg does not affect respiratory enzymes that have nothing to do with mitosis. Fortunately we possess other means of arriving at the same end: F. R. Lillie (1902, 1906) has shown that, if one treats unfertilized eggs of *Chaetopterus* with a mixture of 95% sea water and 5% isotonic KCl, they are stimulated to development; maturation is completed and the egg enters upon a series of monasterian cycles. The chromosomes form

and divide, and, in the meantime, a single aster makes its appearance. It is reformed and the phenomenon is repeated. During the course of these aberrant mitoses the cytoplasm tends to cleave, but the furrows disappear immediately. In this way, a large nucleus packed with chromosomes forms in an unsegmented egg. This nucleus finally ruptures, liberating the chromosomes into the cytoplasm, and one sees a segregation of yolk and of hyaline protoplasm. This latter envelops a central mass of yolk, so that one obtains an undivided egg the composition of which resembles that of the gastrula. Development may continue farther with cilia appearing on the external boundary of the ectoplasm and the egg, still a single cell, resembles the trochophore larva of polychaetes. This interesting

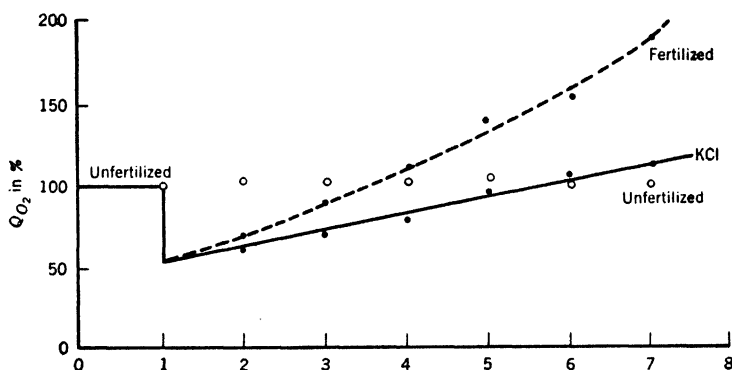


Fig. 32. Respiration of fertilized *Chaetopterus* eggs and of eggs which differentiate without cleavage (KCl); the open circles indicate metabolism of unfertilized eggs (J. Brachet).

phenomenon, termed "differentiation without cleavage" by Lillie, has since been reexamined by Pasteels (1934) and by Brachet (1937).

We have compared the oxygen consumption of the normal fertilized egg of *Chaetopterus* with those that develop without cleavage. As shown in Figure 32, activation by KCl, as in normal fertilization, lowers the rate of oxidations by one half. Then, respiration increases, but at a much greater rate in the fertilized eggs. Control experiments using unfertilized eggs show a constant gaseous exchange with time (shown in Fig. 32 by ○) and KCl used as an activating agent has no effect on the oxygen consumption of fertilized eggs. These facts seem to imply that a part of the respiratory exchange



furnishes the energy for mitosis. However, a closer examination shows that the development of the eggs that differentiate without cleavage is markedly retarded. The cilia form much earlier in the normal trochophores than in the unicellular larvae. Determinations of thymonucleic acid, designed to furnish an indication of the synthesis of chromatin, have established that larvae that differentiate without cleavage contain only 35% of the quantity present in the controls after several hours.

Almost identical results have been obtained by Tyler and Horowitz in *Urechis*. Treatment of unfertilized eggs by 0.01 *N* ammonia for 2 to 7 minutes produces activation followed by differentiation without cleavage, while a prolongation of the treatment for 12 to 17 minutes induces, on the contrary, a true parthenogenesis, which is accompanied by cleavages that are somewhat slower in rate than the controls. It can be seen from Figure 31 that the differences between these two lots of eggs and the controls are very small for the first five hours, after which time the rate of oxidation of the controls rises distinctly faster than that of the haploid eggs. Finally, at the end of 12 to 19 hours, the parthenogenetic embryos are intermediate between the controls and those eggs which differentiate without cleavage.

Andresen, Holter and Zeuthen have recently studied an analogous case in the eggs of a tunicate, *Ciona intestinalis*, in which a few of the eggs after segmentation lose their furrows and form multinucleate masses. The respiration of these masses was followed in the micro-Cartesian diver of Zeuthen (see Chapter I) with the following results: the oxygen consumption of the abnormal eggs is less (20 to 30%) than that of controls and during development, which consists solely of a multiplication of the nuclei, the respiration rises hardly less rapidly than in the normal eggs.

In addition, Fisher and Henry have shown that the sulfamides have an effect similar to that of narcotics on the respiration of the sea urchin egg; they specifically inhibit the fraction of the respiration necessary for cell division such that they stop mitosis although the oxygen consumption is reduced by a very small amount. However, penicillin may, according to dose, retard or inhibit cell division from 0 to 100% without influencing the respiration of unfertilized or fertilized eggs (Henry and Henry).

All of these experiments are unanimous in the recognition of the existence of a relation between the increase in oxidations and

cleavage. The more rapid the cleavage, the more marked the increase in metabolism. The only evidence to the contrary is found in the work of Sugiyama, who found no inhibition of respiration in the eggs of the sea urchin when mitoses were inhibited by aged egg albumin. This isolated observation does not appear to be of such a nature as to modify the conclusions derived from studies on the effects of narcotics and of differentiation without cleavage. But does this increase in respiration during segmentation imply that mitosis needs energy and that mitosis is accompanied by cyclical variations in metabolism? We can not draw these conclusions, since it may well be that the rise in oxygen consumption is simply linked with increase in surface area which results from cleavage. It is clear that this is less in eggs where cytoplasmic cleavage is impeded. Likewise the slowing of development by narcotics and differentiation without cleavage introduces an obstacle to the interpretation of the results, for, if morphogenesis is retarded, it is not very surprising that the normal rise in metabolism is also less rapid. It is clear that the problem is far from simple and that its solution should be sought particularly in investigations devoted to metabolism during the mitotic cycle. Let us examine the following.

## 2. Respiratory Metabolism During Mitosis

In the first place let us consider the interesting work of Ephrussi on the temperature coefficient ( $Q_{10}$ ) during mitosis in the sea urchin egg and in *Ascaris*. In these two cases this coefficient shows large variations (from 1.0 to 2.3) in the different phases of mitosis, from which it is deduced that various processes, some chemical, some purely physical, enter into the different stages of cell division. The values obtained suggest the probable role of oxidation during prophase.

The first attempts to measure gaseous exchange during mitosis go back to 1904 when Lyon followed the  $\text{CO}_2$  production of sea urchin eggs during segmentation and concluded that cyclic changes occurred. The methods employed, however, were too crude in pioneer attempts to be of much significance. The problem was taken up again by Vlès in 1922 on the same material using the spectrophotometer to measure changes in pH produced by  $\text{CO}_2$  in sea water containing the eggs and a pH indicator. The curve obtained (Fig. 33) has a clearly cyclical form.

The first determinations of oxygen consumption during mitosis

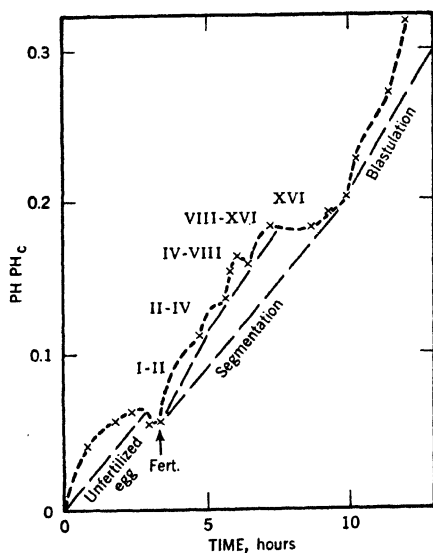


Fig. 33. Variations in pH of sea water containing the segmenting eggs of the sea urchin (Viés). Ordinate is  $pH - pH_c$ .

by the manometric method were performed by J. Gray. They were carried out on the sea urchin egg and gave a perfectly linear curve (Fig. 34). Contrary to his predecessors, Gray did not observe

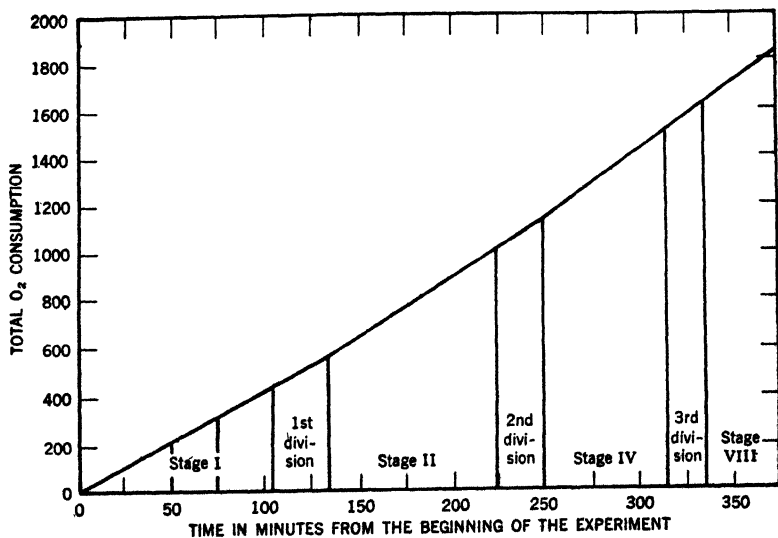


Fig. 34. Oxygen consumption of sea urchin egg during segmentation (Gray).

rhythmic changes in relation to mitosis. He emphasized that the variations observed by Lyon and by Vlès did not agree exactly, the maxima appearing at different stages of mitosis. Gray thinks that the variations could result from changes irrelevant to respiration, such as changes in the alkaline reserve of the egg, the changing pH of the medium or the surface of the egg. One ought not however to exclude, *a priori*, variations in the R.Q. during development. Gray does not reject the idea that the rhythmic production of  $\text{CO}_2$  may be linked with some cyclical anaerobic activity of the egg that results in the formation of this gas. Such a hypothesis is the more plausible since Vlès did not specifically measure  $\text{CO}_2$  but rather the total acid production. His experiments also suffer from the fact that the eggs were not stirred between measurements.

An investigation by Runnström has helped to clarify the situation, since, according to him, the production of  $\text{CO}_2$  recorded by Vlès is in reality the result of acid formation. These acids have their

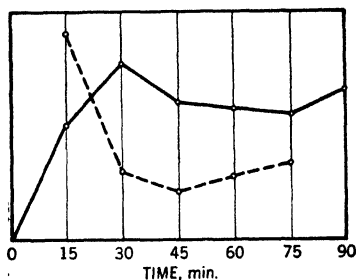


Fig. 35. Solid line = oxygen consumption of fertilized sea urchin egg; broken line = the ratio of free  $\text{CO}_2/\text{O}_2$  (Runnström).

origin in anaerobic reactions, but they are oxidized in the presence of air. The result is that the oxygen consumption is not constant but rises after fertilization and then falls, as had been discovered by Shearer. It then rises again at the time of mitosis, after which it decreases and rises again during the next division. The result is that the sea urchin egg exhibits a series of rhythmic variations in metabolism that are very marked in the case of acid production resulting in the liberation of  $\text{CO}_2$  and less apparent in oxygen consumption (Fig. 35). Runnström does not explain the disagreement between his work and that of Gray, and we must assume that it is either a matter of different species exhibiting a more or less favorable synchronism of development, or that the two investigators worked at different temperatures. It could be that the cyclical

oxidations described by Runnström have a different temperature coefficient from that of the basic respiration of the egg. Thus, Gray, who worked only at 11° C, would not observe them.

The ideas of Runnström have been confirmed indirectly by Reiss, who followed the changes in potential at a platinum electrode near the sea urchin egg during cleavage. He noted a drop in potential after fertilization with a repetition of this drop at each cell division. This abrupt fall of potential could be attributed to variations in the oxygen consumption, to the production of an acid, or to the liberation of reducing substances.

Finally, to be complete, let us add that Tang could not find any variations in oxygen uptake in the egg of the sea urchin *Arbacia*, but his technique has been severely critized by Whitaker, who pointed out that the excessive shaking to which Tang submitted his eggs caused them to cytolize rapidly.

Brachet has studied the frog's egg (*Rana fusca*) (1932, 1934, 1935) from the same point of view. Here the almost perfect synchrony of the mitoses in the same batch of eggs makes them excellent material, but this advantage is offset by a serious difficulty: the abundance of yolk in the egg. This results in a very low respiration, which can only be measured by refined techniques and very complicated manipulations if the determinations are to be made at close intervals. The Warburg manometers lack the sensitivity and were used only for measuring controls. Most of the measurements were carried out with Fenn microrespirometers, which have certain advantages as well as certain difficulties. This type of apparatus gives a high sensitivity, but there is constant danger of obstructions in the capillary, and the temperature must be kept absolutely constant. This latter factor was not controlled to the extent that is desirable. Figures 36 and 37 show the cyclic variations in oxygen consumption which we found in all our experiments (34 for fertilized eggs) and which were lacking in unfertilized eggs. Unfortunately only three experiments were carried out on the latter because they are often cytolized by the vigorous shaking of the apparatus. The fertilized eggs are not susceptible to this injury. The cyclic appearance of the curve is found only when there is a satisfactory synchrony in the mitoses of the individual eggs. Thus, it seems justified to explain the variations of oxygen consumption in connection with mitotic changes. Nevertheless, it is desirable to repeat these in-

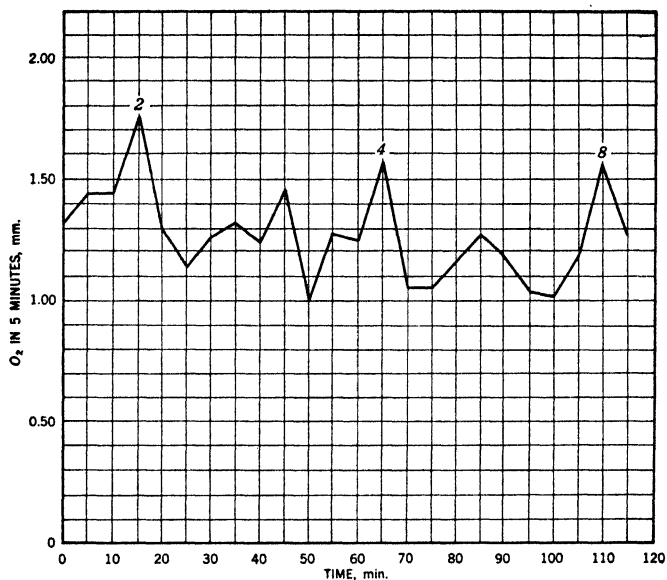


Fig. 36. Oxygen consumption of frog egg during segmentation; the numbers indicate the appearance of furrows (J. Brachet).

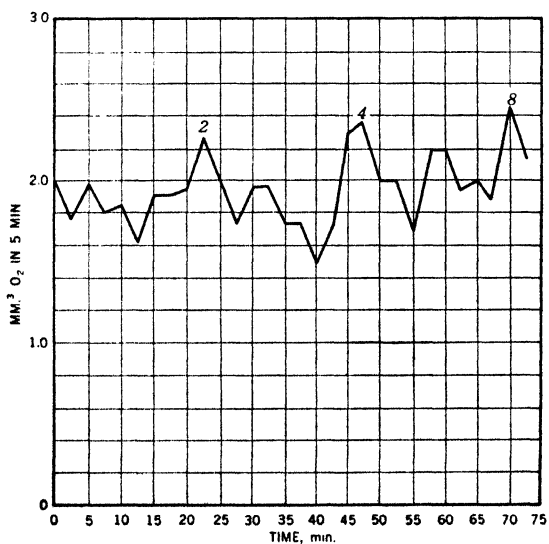


Fig. 37. Oxygen consumption of frog egg during segmentation; the numbers indicate the appearance of furrows (J. Brachet).

vestigations with better technique and more controls. The cytological examination shows that the peaks of the curve coincide with prophase and anaphase, while respiration is low during metaphase and interphase (resting nucleus).

Other authors have taken up the question in connection with other work using similar techniques. Wills observed a regular increase and decrease in the uptake of oxygen, possibly related to mitosis, in one batch of eggs of *Rana pipiens*. Atlas noted that the respirometers he used were not sufficiently sensitive to allow him to come to any conclusions. Privolnev noted a rhythmic variation in the oxygen consumption of the egg of the lamprey with a maximum at the time of appearance of the cleavage furrows, but he gives only three experiments and it does not appear that the method used (Warburg) was sufficiently refined enough; his work, nevertheless, lends some support to the idea of the existence of metabolic differences during mitosis.

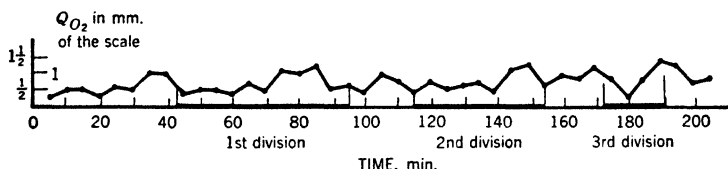


Fig. 38. Oxygen consumption of a single fertilized frog egg during segmentation (Stefanelli).

The analysis was carried a step further by Stefanelli, who measured the oxygen consumption of single eggs of the frog and of the toad (1937). It is clear that the difficulty of asynchronous divisions is avoided when single eggs are used. The Italian investigator, using a respirometer of his own design, concluded that a slight increase in oxidation occurred just before the appearance of the cleavage furrow, and then the respiration fell to its original value. A second peak was observed about ten minutes after the separation of the blastomeres. He found the same behavior during the second and third divisions. The curve then becomes smooth, as we have already seen; this results from the fact that the mitoses after this time are not synchronous in the individual cells of the same egg. Stefanelli came to the same conclusion that we did regarding the presence of cyclic metabolic variations in the metabolism during

the mitotic division. The differences he observed are smaller and it is even questionable as to whether they are not experimental errors (Fig. 38).

Following this type of evidence, we come to an investigation by Trurnit, who has unfortunately published only a preliminary note. This author worked out a technique permitting the measurement of heat production by means of thermocouples only 15 to 30  $\mu$  in diameter. The method of preparing these microthermocouples is not described. Trurnit made some fifty measurements of *Triton* and of sea urchin eggs. In the latter he places 5 to 100 eggs in a small dish filled with a little water in which is immersed a thermocouple by

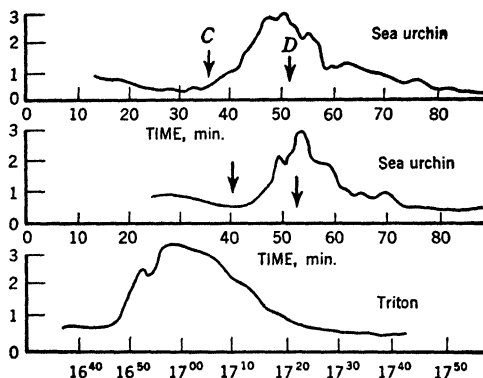


Fig. 39. Variations in the temperature during the division of sea urchin egg and that of *Triton*. One unit of the ordinate corresponds to  $7 \times 10^{-8}$  degrees for the upper two curves, to  $1.2 \times 10^{-8}$  degrees for the lower curve (Trurnit). C, prophase; D, metaphase.

means of a micromanipulator. Observations are made using a binocular microscope within a constant temperature bath and the deflection of a very sensitive galvanometer is recorded photographically so that one can follow the heat production during several successive mitoses. The technique is the same for *Triton* except that the thermocouple is either placed up against the egg or inserted in one of the blastomeres. Figure 39 records the results obtained by this method, which requires very delicate handling. In the two cases studied, Trurnit found an increase in temperature during mitosis, which began in prophase and reached a maximum during cytoplasmic division. This investigator considered his results in connection with those of Runn-



ström and ours and adopts as the most reasonable conclusion the idea that mitosis is accompanied by a greater energy output as compared with preceding stages.

The important problem of the oxygen consumption of the frog's egg has been taken up recently by Zeuthen. The Danish author is not convinced by the research of Stefanelli and Trurnit, since the technique of the former is open to question, while the work of the latter is acceptable only with great reservations—which Linderstrøm Lang and Zeuthen will subsequently discuss. It appeared to them worthwhile to repeat our results by measurements made of a single egg in the Cartesian diver. This instrument, not being sensitive enough in its original form, has been modified by Zeuthen in to a type that contains one frog's egg in a volume of gas of 2 to 3 mm<sup>3</sup>.

Because of the very small size of the gas space the apparatus has a very high sensitivity. But it was not possible to introduce the usual droplet of alkali to absorb the CO<sub>2</sub> into this modified diver and the distinction between O<sub>2</sub> and CO<sub>2</sub> was made by taking advantage of their difference in solubility.

Zeuthen found in all his experiments rhythmical variations in the respiration of eggs undergoing segmentation. These variations are not present in the unfertilized egg, but they are found in eggs in which cytoplasmic division has been accidentally stopped without affecting nuclear division. The variations observed by Zeuthen, after correction made according to some studies by Linderstrøm-Lang on the diffusion phenomena in the diver which was used, are from 7 to 11%, while they reached 25 to 30% in Brachet's experiments. In addition, Zeuthen observed only a single peak during mitosis instead of two. But it must be pointed out that Zeuthen's technique does not tell us whether these variations are concerned with oxygen consumption or CO<sub>2</sub> production. They may even be caused by rhythmic variations in the dissolved oxygen within the egg due to cyclic changes of the permeability of the cell surface to oxygen during mitosis. However, this last possibility is rather remote in light of the fact that the gaseous exchange remains rhythmic in the case of eggs in which cytoplasmic division has been arrested. Also, cyclic variations of the respiration of the same order of magnitude as in the frog have been found in the sea urchin by Zeuthen later on—and in this egg with so little yolk rhythmic changes in the permeability to oxygen would hardly be expected to play any role. Earlier

Zeuthen (1943) measured the oxygen consumption of an isolated egg of *Ophryotrocha* using a microdiver and obtained a straight line. However, in the instrument used the diffusion of gases was slow and it is probable that any possible cyclic variations of respiration during mitosis would be masked for this reason.

We see that the problem is far from simple and that definite conclusions can not be drawn as yet. Nevertheless it seems clearly established that there are cyclic variations of small amplitude in respiration and that these are related directly to mitosis.

### 3. Are Oxidations Necessary for Mitosis?

Early investigators were already interested in finding out whether cell division would occur under anaerobic conditions. Loeb (1894) noticed that the eggs of the fish *Fundulus* survived in a vacuum imposed for four days and that mitosis proceeded for a certain time. But one year later, he announced that cellular division was stopped by anaerobiosis in another fish, *Ctenolabrus*, as well as in the egg of the sea urchin. A little later, Samassa placed the eggs of the frog in a vacuum or in an atmosphere of nitrogen or hydrogen and could follow cleavage up to the blastula stage.

The prompt arrest of mitosis in sea urchin egg has been verified by a number of investigators (Lyon, Harvey, Drastich, Amberson, etc.) The latter has established that the minimal oxygen tension for the continuance of cleavage is 4 mm of Hg. The observations of Samassa on the frog have also been confirmed by several experiments (Godlewski, Bataillon, Parnas and Krasinska, Lennerstrand, J. Brachet, 1934). The egg of *Ascaris*, studied by Samassa, Bataillon, Fauré-Fremiet, Zavadowsky, and Dyrkowska, behaves like that of the sea urchin in regard to lack of oxygen. Thus, the eggs of different species present differences in susceptibility to anaerobiosis, which in some cases does not impede cleavage, but in others quickly stops it.

The addition of cyanide to eggs during cleavage has the same effect as lack of oxygen in that segmentation of the frog's egg is not prevented and development proceeds to the blastula (Bataillon, Bellamy, Brachet). At a concentration of 0.001 *M* (Brachet) about 90% of the oxygen consumption is inhibited. Cyanide, on the other hand, rapidly inhibits mitosis in the sea urchin egg (Blumenthal, Brinley, Runnström 1930, Örström 1932). These last two investigators showed that oxygen consumption is greatly reduced. In

this connection we note the curious fact that the injection of cyanide into the sea urchin egg (Brinley) does not stop mitosis; if this observation is correct it suggests that this poison acts especially on the egg surface. According to Clement, two marine invertebrates (*Ilyanassa* and *Crepidula*) react very differently to cyanide treatment, for the cleavage of the former continues in the presence of this substance, while in the latter it is quickly stopped. The necessity of oxidation in relation to mitosis thus varies considerably in different species.

Apparently similar phenomena are obtained with the cells of older organisms, since the growth *in vitro* of fibroblasts is stopped suddenly under anaerobic conditions (Wind, Ephrussi, Chevillard, Mayer and Plantefol, Lipmann) although lack of oxygen does not prevent the migration of cells, according to Ephrussi and his collaborators. Laser, on the other hand, has obtained growth *in vitro* of several types of cells in the total absence of oxygen and attributes the results of other investigators to the toxic effect of the vapors of the phosphorus that was used to get rid of the last traces of oxygen. According to Voegtlin and Chalkley, division of ameba is not prevented by carbon monoxide, although cyanide and hydrogen sulfide tend to suppress the prophase. Thus, mitosis in the Protozoa does not appear to have a direct relation to oxidation.

There are several interesting studies on the cytology of eggs subjected to anaerobiosis and cyanide. In the sea urchin egg the asters disappear in the absence of oxygen and reappear upon the introduction of this gas (Loeb). Inhibition of respiration prevents the swelling of the nucleus and the breakdown of the nuclear membrane (Runnström 1930) but has little influence on the later phases of mitosis (Förster and Örström). The asters become more distinct in the presence of cyanide, which does not prevent the formation of the spindle when oxidation is inhibited to the extent of 85%. Runnström concludes from his experiments that it is the cytoplasmic division that is most susceptible to inhibition of oxidation. Growth and breakdown of the nucleus come next and contrary to the claims of Loeb, the formation of asters and of the spindle does not require a high respiration.

In the case of the frog, at the time when cleavage is arrested, we have found various anomalies, such as degeneration of the chromosomes, of the spindle, and of the asters, partial rupture of the

nuclear membrane, etc. It is difficult to say in this case which phase of mitosis is particularly susceptible to anaerobiosis.

The situation is not the same in *Ascaris*, where Bataillon always found a blocking of the nucleus in the resting stage or in metaphase, when the eggs are deprived of oxygen. This would imply that the stages following, prophase and anaphase, require high rates of oxidation. It must be remembered that these are precisely the times when respiration appears to increase in amphibians and, as a result of these observations of Bataillon, it would be very interesting to measure by modern techniques the oxygen consumption in *Ascaris* during mitosis. Finally, in *Chaetopterus*, cyanide reduces the size of the asters and interferes with the division of the chromosomes as well as cytoplasmic division (Brachet, 1937).

Some investigators have attempted to substitute the oxygen of air with other substances. Reiss and Vellinger, working in absence of air, placed sea urchin eggs in substances capable of transferring their oxygen rapidly such as oxyhaemoglobin and oxyhaemocyanine, with the result that segmentation took place. Subsequent studies showed that development could occur only if the medium was neither strongly oxidant nor strongly reductant. The upper and lower limit of the oxidation-reduction potential that permitted development was termed the "potentiel d'arrêt" (Reiss). Various oxidants blocked cleavage when their potential passed this critical value regardless of their chemical composition. Finally, according to Rapkine, development of the sea urchin egg is possible under anaerobic conditions if methylene blue is added, but this result could not be confirmed by Örström (cited by Runnström, 1933). The latter obtained negative results on addition of pyocyanine, which should act like methylene blue (1935). It seems, then, that in the sea urchin egg, at least, oxygen can not be replaced by another hydrogen acceptor.

What emerges from all these experiments is that certain eggs absolutely require oxygen while others can act for some time as facultative anaerobes. The frog's egg undoubtedly belongs to this second class, for mitosis is possible under complete anaerobiosis or in the presence of cyanide, which inhibits oxidation to the extent of 90%. Admittedly, then, if there are cyclic variations in metabolism during cleavage in this species these are of only secondary importance, epiphenomena which can be suppressed without damage but which may constitute evidence for cyclic anaerobic reactions. It

may be that, as in the case of the sea urchin egg (Runnström 1933), substances form under anaerobic conditions which can be oxidized by respiration. If this is the case, one can more easily conceive of the differences that exist between various species, for, depending on whether the products of anaerobic reactions are more or less abundant or more or less toxic, mitosis will be stopped suddenly or slowly. In the frog, as we shall see later, oxidation continues for some time under anaerobic conditions by means of an oxidative reserve and thus it is seen that the toxic products of metabolism do not accumulate very rapidly. We do not know whether this respiration without oxygen takes place in the sea urchin egg, but the rapid production of acids in anaerobiosis makes it improbable. The study of the anaerobic metabolism of these two types of eggs is, consequently, very desirable, in spite of the technical difficulties encountered.

Let us end this discussion with several observations by Clowes and his collaborators who further emphasize the independence of cell division and oxidation in the egg of the sea urchin, which requires oxygen for cleavage. Clowes and Krah1 (1936) studied the action of various nitro and halogen derivatives of phenols on mitosis. These substances *increase respiration* but reversibly *block* mitosis at prophase. The concentration for stopping development is about the same as that which gives maximum respiration. These authors are of the opinion that a complex is formed between the phenolic derivatives and some nitrogen-containing substance that interferes with both metabolism and cleavage; whether these derivatives act in the form of ions or nondissociated molecules is a controversial point (Krah1 and Clowes, Tyler and Horowitz, 1938). However, there is agreement that mitosis is reversibly blocked when the optimum concentration for stimulation of respiration is exceeded. Thus, the block occurs when the cells are still actively respiring. In addition, Krah1, Keltch, and Clowes have tried the effects of various inhibitors of metabolism on mitosis by controlling their influence on respiration and have come to the conclusion that neither cytochrome nor copper have a role in mitosis. Still more recently, Krah1, Keltch, Neubeck and Clowes have noted that cell division is stopped in the sea urchin egg in the presence of  $\text{NaCN}$ ,  $\text{NaN}_3$ , and  $\text{Na}_2\text{S}$  in doses which inhibit respiration 50%; these agents are also known to combine with cytochrome oxidase. All these facts taken together show that there is no direct relation between the degree of oxidation and the maintenance

of mitosis. One must remember, however, that there are several indications in favor of the idea that a specific fraction of the respiration, sensitive to narcotics and to sulfamides, may be necessary for cell division (Fisher and Henry, Moog).

A valuable suggestion of the nature of the chemical reactions that form the basis for mitosis is found in a recent work by Spiegelman and Moog on *Rana pipiens*. These authors have found that, contrary to the earlier observations by Barnes, segmentation is stopped by traces of  $\text{NaN}_3$  while cyanide has no effect as in *Rana fusca*. Now, we know from Keilin's work that sodium azide, like cyanide, inhibits cytochrome oxidase but, since cyanide does not affect cell divisions, we conclude that an enzyme sensitive to azide but not to cyanide is essential to segmentation. It may be, as Spiegelman and Moog suggested, that it is an adenylypyrophosphatase (apyrase), which we know is azide-sensitive but not cyanide-sensitive (Meyerhof, Moog and Steinbach). Thus it would be interesting to make a further study of the phosphate metabolism of the frog's egg during division, both under aerobic and anaerobic conditions, to see if there are any rhythmic changes. The arrest of mitosis by azide has also been noted by Fisher and Henry in the sea urchin.

The unusual experiments of Beams and King and of Huff and Boell may be noted here. The former centrifuged the eggs of *Ascaris* at the tremendous force of 400,000 g. for one hour without interfering with cell division although a marked stratification of the cytoplasm was produced. Mitosis is thus possible at speeds at which the proteins sediment out *in vitro* and it is concluded that the constituents of the hyaloplasm are firmly bound to each other. According to Huff and Boell respiration of the centrifuged eggs decreases 75%; nevertheless, mitosis is normal. The inhibition pertains exclusively to the fraction of oxidations that is sensitive to cyanide and therefore catalyzed by cytochrome oxidase. This is easily understandable now that it is known that this enzyme is bound to the granules which, under ultracentrifugation, pack down at the centrifugal pole of the cell (Stern, Chantrenne). Thus one can reduce the activity or modify the localization of these granules and consequently the oxidations without altering mitosis.

The study of oxidations in the cell during mitosis has brought to light some interesting facts, but it has not revealed any general idea that helps us to understand the detailed mechanism of cell di-

vision. It must be admitted that attempts in this direction up to the present have been somewhat disappointing and we have seen the same for the relations between metabolism and fertilization. Let us see if the examination of other chemical properties of the cell yields more satisfactory results.

#### 4. Nonrespiratory Metabolism of the Cell During Mitosis

Among the changes taking place in the cell during mitosis, special significance is attached to those involving —SH groups. The role of these radicals in cell division has been especially emphasized by Hammett, Rapkine, and by Voetglin and Chalkley.

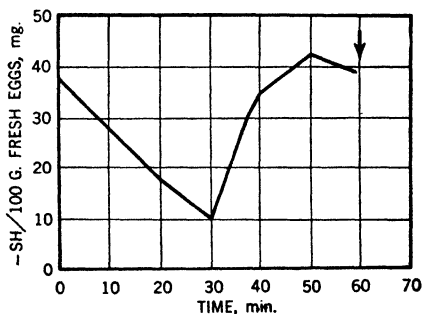
Hammett ascertained that the growth of roots is prevented by addition of lead salts and that this inhibition is overcome when substances containing the —SH group (thiocresol, thiophenol, cysteine, etc.) are added. From this it is deduced that lead combines with the —SH radicals of the cells and that this binding produces the arresting in growth. In addition, the root tip, where cell divisions are most numerous, shows a particularly intense nitroprusside reaction. The addition of substances rich in —SH groups should accelerate regeneration and growth and the best case seems to be that of ameba (Voetglin and Chalkley), where reduced glutathione stimulates an increase in nuclear volume often followed by division. It should also be mentioned that, according to Firket and Comhaire, the amount of glutathione and the intensity of the nitroprusside reaction increases considerably during the germination of the pea, a conclusion that was recently confirmed by Hopkins and Morgan. Analogous results have been obtained still more recently in Hammett's laboratory by Miller and Reimann who find that cysteine speeds up cleavage of mammalian eggs *in vitro*, while Coldwater, Owen, Weiss, and Prince showed that reduced glutathione favors growth and regeneration in planarian worms. In addition, Riley reports that cysteine has a beneficial effect on the healing of wounds. We shall return later to these observations in relation to cells cultivated *in vitro*.

It is important to note, however, that certain investigators have not obtained positive results using substances containing —SH groups. For example, Gaunt observed no stimulation of mitosis on addition of cysteine to the gastropod eggs of *Physa* and *Limnaea* and Sun found the same in the sea urchin egg. Morgulis and Green have obtained no acceleration of regeneration in a polychaete

(*Podarke obscura*) after section and addition of thiocresol, thiophenol, thioglycollic acid, and cysteine. These negative results are perhaps open to question on the basis of a failure of the substances to penetrate into these cells. It is also possible that some of these sulfhydryl derivatives are toxic, as is the case with cysteine for the sea urchin egg, according to Lindahl, as well as Hörstadius and Stromberg.

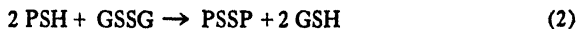
The investigations of Rapkine are worthy of a more lengthy treatment because they have led to the development of definite hypotheses regarding the chemical basis of mitosis. Rapkine (1931) studied the amount of —SH groups in the sea urchin egg during cleavage and as noted earlier (Chapter IV) the amount of reduced glutathione decreases just after fertilization as a result of the oxida-

Fig. 40. Variations in the content of reduced glutathione in sea urchin egg from fertilization (zero time) up to division (indicated by arrow) (Rapkine).



tion of the sulfhydryl to the disulfide. Later the amount of reduced glutathione rises again to attain a maximum shortly before the cell division (45 minutes after fertilization); this is a result of the reduction of the oxidized glutathione (Fig. 40).

What corresponds to these variations in the proportion of the oxidized and reduced states of glutathione? Hopkins demonstrated that reduced glutathione is able to reduce the disulfide group (—SS—) of proteins, to the —SH group, and, *vice versa*, oxidized glutathione can oxidize the —SH radical of proteins. These reactions may be written in the following way:



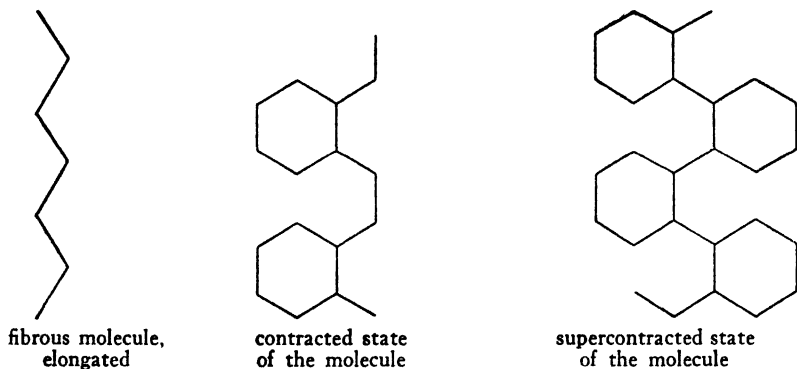
where P = protein and G = glutathione.

It is seen that a direct relation exists between the proportion of reduced and oxidized glutathione and the degree of oxidation of the



sulfur bonds in proteins. These reactions have been utilized to increase (1) or to decrease (2) the amount of —SH groups of the proteins. Certain enzymes are inactivated by oxidized glutathione because the —SH radical is necessary for their function; this is the case especially with triosephosphate-dehydrogenase (Rapkine), with cathepsin and some peptidases (Bersin), with succinic dehydrogenase (Hopkins and Morgan), with urease (Sumner), and with choline esterase (Nachmansohn); on the other hand, insulin (Stern and White) and prolactin (White) will only act if their sulfur is in the —SS— form and reduced glutathione will thus inactivate them.

Rapkine believes that reactions of the above types play a role in the egg: indeed, the oxidation of reduced glutathione after fertilization will occur even in the presence of cyanide, so there must



be an oxidation at the expense of the —SS— proteins after reaction (1). The increase of reduced glutathione in the egg just before division should come from the reverse reaction (2).

Now, we know from the work of Harris that the denaturation of a variety of proteins is accompanied by a liberation of —SH groups; the phenomenon is explained in the following manner. The native protein is in a globular form with a partially cyclic structure of the molecule. Some of the —SH groups are enclosed by the ring and thus do not take part in reactions involving these radicals. Denaturation would have the effect of opening these rings: the molecule elongates, taking on a fibrous character, with the —SH groups coming to the surface and so becoming reactive. The above diagram by Astbury illustrates these changes.

Finally, it is seen from the work of Anson and Mirsky (see Mirsky 1938) that denaturation is a reversible phenomenon under physiological conditions. It is met with especially in the fertilization of the sea urchin egg, in muscular contraction and in the reception of light by the retina.

A number of these facts were not known in 1931 and thus it is the great merit of Rapkine that he interpreted the variations in amount of —SH groups which he found in the egg to a reversible *denaturation of the proteins during mitosis*. This denaturation would precede division and would be accompanied by an increase in the amount of free —SH radicals of the egg proteins. The —SH groups of the proteins would then reduce the oxidized glutathione according to reaction (2). These changes would have further effects on the carbohydrate metabolism of the egg. This fact, which was only suspected in 1931, seems the more likely since the recent work of Rapkine (1938) showing that triosephosphatedehydrogenase, one of the most important enzymes in carbohydrate breakdown, ceases to function when the —SH groups are oxidized or blocked. Indeed, the lactic acid content of the egg increases little by little to attain a maximum about 10 minutes before division. The colorimetric method used is unfortunately not sufficiently refined, although it is significant that Zielinski has subsequently upheld the idea that the quantity of lactic acid in the egg changes during mitosis. The latter's analyses, although few in number, indicate a 50% decrease from metaphase to the appearance of the cleavage furrow, after which the concentration of lactic acid increases.

Rapkine has other arguments for the support of his thesis. He has observed, in collaboration with Chatton and Lwoff, some particularly striking phenomena in the protozoan, *Gymnodinioïdes inkystans*, which does not contain any —SH groups detectable by the nitroprusside reaction during a large part of its life cycle. At the moment when the yolk begins to break up, the reaction becomes positive and its intensity increases progressively, attaining a maximum just before division. It decreases at the time when the furrow appears. In favorable cases a new increase in color is observed just before the second division, followed shortly by a decrease. Thus in this infusorian as in the sea urchin egg, the concentration of the —SH groups reaches a maximum just before division.

Finally, Rapkine has noted that dilute  $\text{HgCl}_2$  blocks cell division

in the sea urchin egg and that this blockage is removed by transferring the eggs to sea water containing sulfhydryl compounds such as cysteine or thioglycolic acid (Fig. 41). It seems plausible that the arrest of mitosis may be a combination of the mercury with the —SH groups of the egg. It is well to recall here that the antiseptic or at least bacteriostatic action of  $\text{HgCl}_2$  also results in a blocking of the —SH group, and that growth is resumed in the presence of cysteine or reduced glutathione (Fildes).

Rapkiné has since taken up the question of the role of —SH groups in the growth of yeast where the blocking of these radicals

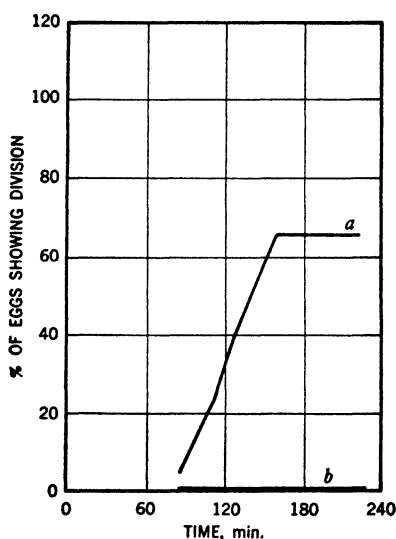


Fig. 41. Sea urchin eggs treated for 5 minutes with  $M/100,000$   $\text{HgCl}_2$ , 40 minutes after fertilization: (a) placed in sea water plus cysteine; (b) placed in pure sea water (Rapkiné).

is effected by means of monoiodoacetic acid, the specificity of which is now well established (Rapkiné, 1933; Dickens, 1933). The penetration of this inhibitor in the cells can be followed quantitatively by the rate of glycolysis, which undergoes a rapid inhibition. Rapkiné has, in addition, studied the effects of iodine, which oxidizes the —SH groups to —SS— groups, but where the action is naturally less specific. Monoiodoacetic acid and iodine stop growth and here again the inhibition is stopped when the cells are placed in a solution of reduced glutathione. The author deduces from this that the hypothesis which states that there is in the predivision stages a denaturation accompanied by an increase in protein —SH and soluble

—SH groups accounts for the effects produced by the two inhibiting agents used in the experiments.

This hypothesis gains support from the investigations carried out on cells cultivated *in vitro*. Ephrussi observed that the nitroprusside reaction became negative as the growth of the cells stopped, even when the cellular proteins were denatured by heat. On the contrary, heating of the cells during active growth brings about an increase in the intensity of the reaction. The arrest in growth, then, would be a result of the exhaustion of the —SH groups of glutathione and of the proteins. These results have recently been confirmed by Ruffili, who found that the reactivity of the —SH groups disappeared in cultures in which development was blocked by the addition of cyanide or hydrazine hydrate and became positive again when growth was resumed. The Italian investigator believes that the changes in the concentration of the —SH groups are consequences rather than the causes of mitosis, but this point seems very difficult to establish and it can only be claimed that the two phenomena are closely related. Finally, Monroy and Leone did not obtain any color with the nitroprusside reaction in resting cells cultivated *in vitro* while the reaction became positive when the cells began to divide.

It is known that the growth of cells *in vitro* is stimulated by chick embryo extract, and Sachs, Ephrussi, and Rapkine have established that this extract is rich in —SH groups. Besides, Verne and Verne-Soubiran have observed that if one inactivates this extract by heat, the addition of glutathione will partially reactivate it. Sulfhydryl compounds also stimulate the proliferation of cells cultured *in vitro*, according to Baker. Finally, we owe to A. Fischer (1940) a detailed study of the effects of amino acids—added singly or in mixtures—on growth *in vitro*. He finds that cystine is especially important and relates this fact to the conclusions of Rapkine and Ephrussi. In addition, he notes that the denatured proteins with a higher concentration of —SH groups are more active than native proteins. A more recent study by Astrup and Fischer leads these Danish authors to the conclusion that glutathione forms a factor in the growth of cultures but that it alone does not suffice to insure growth. However, it is necessary to add that the older work of Huepner and his collaborators has led to conclusions opposite to those of Baker, Verne, and Fischer.

Several attempts have been made to determine the presence of —SH groups during mitosis of the cell. We have just seen the results of Monroy and Leone on fibroblasts cultured *in vitro*. Chalkley has studied ameba, in which the nucleus is particularly rich in —SH groups and in which, during anaphase, the cell becomes uniformly colored by nitroprusside with the exception of the chromosomes. Within the nucleus the reaction becomes more intense from telophase up to the prophase of the following division. Enucleation does not influence the reaction, but it becomes weaker in dying cells.

Unfortunately, it is not possible to eliminate completely a denaturation of the proteins by the reagents used and it is clear that this interesting question should be reexamined in the future by using different methods. For this reason, we only mention some of our own observations (1940) on fixed amphibian eggs and insect testes in which the nuclear sap, the spindle, and the asters are characterized by a particularly high concentration of —SH groups in their proteins after denaturation.

Let us add that Chapman and Hammett and Chapman have obtained a strong reaction of the —SH groups in the hydroid *Obelia*, especially during active growth, and these investigators attempted to reduce the danger of denaturation as much as possible.

One group of investigators has examined, like Rapkine, the effects of moniodoacetic acid on mitosis. Ellis found no inhibition of cleavage of the eggs of *Urechis* and of the sea urchin with 0.01*M* and 0.001*M* solutions, although the substance penetrated the eggs, causing a 60% reduction in the concentration of glutathione. These facts led him to deny the participation of —SH groups during mitosis. But Rapkine has justly pointed out (1937) that the —SH groups which are indispensable for mitosis are apparently those in the proteins, and as Rapkine himself showed the glutathione reacts faster with the moniodoacetic acid than do the proteins. Thus, it is evident that under the conditions of Ellis' experiment the blocking of the —SH groups of the proteins must have been very slight. Runnström (1935) used a more concentrated solution (1/30*M*) on the sea urchin egg and described a marked slowing of development. He noted a tendency toward a separation of the blastomeres which led him to assume an action on the cell membrane. In one case he obtained a blocking at the first division (at prophase) in 95% of the eggs. Zielinski has utilized on the same material still higher

doses (0.025M to 0.15M) with a very rapid stopping of mitosis. The phosphate compounds of the egg were changed as they are in muscle which has been poisoned with monoiodoacetic acid.

Brachet has not succeeded in preventing segmentation in the eggs of the frog or of *Triton* by the use of this substance, even in a 1/30M concentration (1934, 1939), but this failure is probably a result of a lack of sufficiently rapid penetration into the egg. Unpublished observations made in collaboration with L. Rapkine have definitely shown that iodoacetamide, which penetrates the egg rapidly, stops cleavage; moreover, this substance reacts faster with the —SH groups than the corresponding acid. Chloropicrin ( $\text{CCl}_3\text{NO}_2$ ), which, according to Bacq, shows a great affinity for —SH groups, rapidly stops mitosis in the eggs of amphibia and as in the sea urchin egg there is a tendency for the blastomeres to separate. In addition, a rapid degeneration of the asters and the spindle occurs without any visible deterioration of the chromosomes. This observation may be related to the intense nitroprusside reaction that occurs after fixation in the cell membrane and in the achromatic figure of the egg. In weak concentrations chloropicrin stops cytoplasmic division, although karyokinesis takes place in a normal fashion. In the case of the egg of *Rana pipiens* cleavage can be blocked by monoiodoacetic acid and its amide (Pomerat and Haring; Barnes).

Furthermore, Pomerat and Willmer report that monoiodoacetate, even in dilute concentrations, quickly prevents the growth of cells cultured *in vitro*. They do not discuss the role of —SH groups during mitosis and attribute the arrest of proliferation to a suppression of carbohydrate metabolism which is also obtained by the addition of fluoride and glyceraldehyde which interferes with carbohydrate breakdown. The specific inhibitors of cytochrome oxidase (KCN, CO,  $\text{NaN}_3$ ) act much more slowly on growth. Pomerat and Willmer put forth the hypothesis that growing cells exhibit *glucolysis*, in which they break down glucose directly in place of glycogen. Glucolysis is distinguished from glycolysis by the fact that phosphorylation does not play a role in the former. The arguments of these investigators are not convincing, since they have not demonstrated by analytical methods the existence of glucolysis in their material; furthermore, the reality of this phenomenon has been questioned by Meyerhof and Perdigon, as we shall see later. In a subsequent

paper, Willmer came to the conclusion that there is no direct relation between the rate of growth *in vitro* and either glycolysis or glycolysis.

The necessity of —SH groups for the proliferation of cells cultivated *in vitro* is also shown by some research on the action of chemical warfare poisons which combine with sulfhydryl groups; thus, lewisite blocks growth of tissue culture *in vitro* by reacting with the —SH group; the addition of BAL (British antilewisite or 2,3-dimercaptopropanol) allows growth to begin once more (Peters, Stocken, and Thompson). A selective inhibition of growing regions has been reported by Gillette and Bodenstein upon subjecting the larvae of *Triturus* to the action of bis-chloroethylmethylamine chlorhydrate. Chemical warfare poisons also inhibit the division of the sea urchin egg. Finally Briggs has noted that hexenolactone, which combines with —SH groups, rapidly stops cytoplasmic division in the segmenting frogs egg.

Examination of the investigations considered above leaves hardly any doubt of the fact that —SH groups are necessary for cell division. Naturally, it is too much to believe that they are the only factors in mitosis or to think that the hypothesis of Rapkine regarding the denaturation of the proteins during cell division has been as yet demonstrated. Nevertheless, this theoretical concept appears to be very attractive because it permits us to explain certain characteristics of mitosis. It is known that the viscosity of the cell protoplasm changes during division, and in the sea urchin egg a maximum is obtained at prophase followed by a progressive decrease up to interkinesis. It is also generally admitted that mitosis is accompanied by reversible colloidal changes such as the change of the sol state to the gel state. The gelation of the cortex may be the cause of the formation of the cleavage furrow (Marsland). But the denaturation of proteins (see Mirsky 1938) is correlated with a loss in solubility and an increase in viscosity. A still more important point is that in denaturation a globular protein is transformed into the fibrous state, certainly characteristic of the spindle and the asters. These structures are labile and disappear at telophase and it is tempting to think as Rapkine does that they are the result of a reversible denaturation of certain proteins. In addition, the spindle in the living cell is birefringent, as has been shown by Runnström, Schmidt, Moore, and Miller, and thus it seems neces-

sary to conclude that it is composed of elongate molecules oriented in a parallel fashion resembling the structure of fibrous proteins. Furthermore, Schmidt has established that the median part of the spindle becomes isotropic at anaphase, while the portion of the spindle between the chromosomes and the asters remains birefringent. The migration of the chromosomes toward the poles can be understood in the light of Rapkine's hypothesis and the contraction of the spindle fibres would result in the return of the molecule to the native state, *i.e.*, a transformation of fibrous molecules into globular molecules.

Let us recall here that proteins become denatured when they are made into monomolecular films; could it not be that modifications of this sort are produced at the surface of the cell membrane and might these not be the origin of the colloidal changes which Marsland thinks are responsible for cytoplasmic division? This is evidently still only an hypothesis, but it merits consideration.

It is seen that Rapkine's hypothesis becomes particularly stimulating when we consider it as we have just done in relation to modern discoveries on the denaturation of proteins. It permits, in effect, the formulation of the problem of mitosis on a tangible basis amenable to experimentation. The final answer will naturally depend upon the results of the latter.

The importance of sulfhydryl groups during mitosis should not obscure the fact that other chemical substances have a beneficial effect, notably the *nucleic acids* and some of their derivatives. Ugata has observed a clear-cut stimulation of the division of *Paramecium* by addition of nucleic acid and the activity appeared to be associated with the pyrimidine components of this acid. More recently, A. Fischer (1941) and A. Fischer and Astrup reexamined the problem of the growth of cells *in vitro* and tried to determine the nature of the active substances present in chick embryo extract. After experiments on filtration, centrifugation, heating, etc., Fischer came to the conclusion that only the protein fraction is effective. Furthermore, the extract is inactivated by digestion with pepsin or trypsin. But if one tries to isolate the substances stimulating growth from various organs, one finds that only the *nucleoprotein* fraction is really active, while the globulins and albumens do not favor growth *in vitro*. The addition of nucleic acid or denatured nucleoprotein has no effect whatever, so the conclusions are that the important fac-



tor is the complete nucleoprotein and that the nucleic acid and the protein taken separately are ineffective. Almost identical conclusions were reached by Davidson and Waymouth after extended research into the nature of the substances present in pancreatin and various animal tissues which stimulate growth *in vitro*. Here again it appears to be a matter of nucleic acids or their derivatives.

By ultracentrifugation of embryonic extract a layer of granules rich in nucleoproteins is obtained which stimulates growth (Tennant, Liebow, and Stern). However, the supernatant liquid is also active, but Brachet and Jeener have shown that it contains ribonucleoproteins. More recently, Tennant, Stern, and Liebow have shown that purified nucleic acids of yeast, the thymus, the pancreas, and sperm stimulate growth *in vitro* even in low concentrations. In this connection, note that Schultz has recently reported that the addition of ribonucleic acid or adenylic acid to a synthetic nutrient medium results in a considerably accelerated growth in *Drosophila* cultures and on the contrary thymonucleic acid exercises an inhibiting effect.

These facts should be considered together with the observations of Loofbourow and his collaborators, whose experiments were done on the growth of yeast in chemically determined media. The addition of yeast juice, as well as of a brei of chick, rat embryos, or adult *Triton* first irradiated with ultraviolet light, to this medium gave an acceleration of growth. The extracts retained their activity after heating or passage through a Berkfeld filter, and when concentrated yielded a substance rich in phosphorus and with an ultraviolet absorption similar to the purines; probably, it is either a mono- or dinucleotide. In addition, a favorable effect on the growth of yeast is obtained by adding to the culture adenosine triphosphate, muscle adenylic acid, or adenosine while yeast adenylic acid and thymonucleic acid are not active (Loofbourow 1942). The results of Loofbourow were confirmed by the experiments of Davidson, who demonstrated that irradiated yeast lost derivatives of yeast nucleic acid to the external medium. A favorable action of nuclear debris rich in thymonucleic acid on the frequency of mitosis in regenerating liver was noted by Marshak and Walker. Nucleic acid products also stimulate the growth and regeneration of hydroids according to Hammett and his students; in this case the amino acids produce a similar effect. In yeast, on the contrary, Loofbourow has tried in vain some fifteen amino acids. Finally, the recent investigations of

Mac Ilwain show that derivatives of acridine (trypaflavine, etc.) inhibit bacterial growth and combine with nucleic acids; it is only necessary to add some of the latter to stimulate growth again. A number of investigations (Fildes, Woolley, Schöpfer, Kidder, von Euler and Jaarsma, *et. al.*), which we can not treat in detail here, have established the necessity for adding nucleic acids or certain of their derivatives in order to obtain optimal growth of bacteria or protozoa.

At this point we will discuss the carcinogenic agents which are, in general, cyclic hydrocarbons of a sterol nature. When these substances are applied to the skin a tumor develops after a certain time. However, these substances do not stimulate mitosis and often cause abnormal cell division. According to von Möllendorff and Töndury, ordinary sterols and the sterol-like sex hormones cause both a slowing of divisions and abnormalities in mitosis of cells cultured *in vitro* and in the cleaving eggs of amphibians. Keltch, Krahl, and Clowes, however, have described a slight stimulating action of carcinogenic agents on the cleavage of sea urchin eggs, while Calcutt observed an acceleration of the growth of the infusorian *Colpidium* by addition of oestrone to the culture.

Colchicine can also be classified among the "mitotic poisons." The activity of this substance was discovered by one of A. Dustin's students, Lits, who showed that mitosis was blocked in metaphase by a degeneration of the spindle. In plants the poisoned cells may recover, and this treatment produces polyploidy (Blakeslee). According to Dustin, colchicine stimulates cells which are in predivision stages to enter mitosis, but this opinion is not generally accepted (see Verne and Vilter). In the sea urchin egg, colchicine inhibits the process which is responsible for the rhythmic changes in viscosity during mitosis (Beams and Evans; Wilbur); comparable results have been obtained in *Tubifex* eggs by Lehman and his students. Because of the possible relation of changes in viscosity to denaturation of the egg proteins a study should be made of the relation of this latter phenomenon to the effects of colchicine. The hypothesis of an action of colchicine on the fibrous proteins of the spindle has been developed in an interesting paper by Östergren.

Colchicine and carcinogenic agents are not the only poisons of mitosis. Arsenic, trypaflavine (Dustin) and, according to Lettré and Fernholz, all substances containing the sympathomimetic group

(benzene ring with attached carbon and amino group) belong to this class of substances.

The mode of action of carcinogenic substances on the cell is not well known, although some recent investigations furnish valuable clues which give rise to some striking comparisons. For example Wood and Fieser have established that the carcinogenic derivatives of benzantracene and benzopyrene form condensation products with cysteine and other sulfhydryl compounds. They arrive at the hypothesis, which they subsequently retracted, that the carcinogenic compound breaks a disulfide bond ( $-\text{SS}-$ ) present in the protein of the normal cell, thus forming the sulfhydryl derivative of the hydrocarbon. This idea appears to be related to the curious observations of Berenblum showing that the skin of adult mammals no longer reacts to carcinogenic hydrocarbons when it is treated with yperite and that this toxic gas, like all those causing blisters, combines rapidly and selectively with  $-\text{SH}$  groups, as shown by Bacq. Similar results have recently been obtained by Crabtree and by Reiman. All these facts are understandable if the primary action of carcinogenic agents is concerned with the  $-\text{SH}$  groups of proteins. The blocking of these radicals prevents the combination of the protein with the carcinogenic compounds. It is easy to see that the reaction between these substances and  $-\text{SH}$  groups would result in a slowing of division and in mitotic abnormalities if the sulfhydryl proteins play the role in cell division attributed to them by Rapkine. Recent investigations (Kensler and collaborators; Potter; Potter and DuBois; Elson and Hoch-Ligeti) have confirmed in interesting fashion the idea that derivatives of carcinogenic agents block the  $-\text{SH}$  groups of the proteins. These authors have obtained inhibition of a number of enzymes (triosephosphatedehydrogenase, succinic dehydrogenase, urease) in which the presence of the  $-\text{SH}$  group is necessary for functioning by adding the products of oxidation of the carcinogenic hydrocarbons. Note further, along these lines of thought, that Lieberman has recently demonstrated that cysteine reacts with certain sterones with the ketone group in 3 positions; the author suggests the possibility that certain steroids can combine with the  $-\text{SH}$  groups of the proteins or enzymes in such a manner as to form a prosthetic group.

The hypothesis just outlined can perhaps be pushed still further. Graffi has recently followed the fate of benzopyrene in the cell with

the fluorescent microscope and to all appearances this hydrocarbon accumulates in the granules which are rich in ribonucleoproteins. The pictures published by Graffi in the case of yeast are particularly suggestive in this regard. If one isolates these granules by homogenizing and ultracentrifuging and adds benzopyrene to them, they bind it rapidly. We will elaborate on the physiological role of these granules in the following chapter, but suffice it to say here that they contain ribosenucleic acid associated with sulfhydryl-containing proteins and that they stimulate growth *in vitro* according to A. Fischer and Tennant, Liebow, and Stern. Furthermore, these granules, oddly enough, resemble in their physical and chemical properties the pathogenic viruses (Claude, Levaditi). It is especially interesting to know that the carcinogenic hydrocarbons accelerate and intensify the effects of certain viruses, for example, that of the papilloma of Shope (Peyton Rous and Friedewald). From this assemblage of facts it appears that the carcinogenic substances combine with particles in the cell which resemble viruses and which contain —SH groups and nucleic acids and we know that these latter substances play an important role in mitosis.

Similar ideas have just been developed by Potter (1944), who offers the hypothesis that cancer is caused by a blocking of the —SH groups in one of the oxidative enzymes which form a part of the composition of the ribonucleoprotein granules. The granule, thus changed, is transformed into a carcinogenic virus. Inhibition of the —SH groups of respiratory enzymes is brought about by the carcinogenic hydrocarbons. Certain cytological observations of Woods and DuBuy suggest that perhaps it is at the surface of the mitochondria where one must look for the normal granule capable of being transformed into a virus.

Is there any reason to believe that the granules displaced by ultracentrifugation play a role in mitosis? It would seem that there is, for A. R. Moore has called attention to the direct relationship of the granules which can be moved by centrifugation and the division of the egg of *Dendraster*. If one submits the unfertilized egg of this species for about ten minutes to a force of 40,000 g, the granules sediment to the centrifugal pole while the nucleus moves to the centripetal pole. If then the parthenogenetic treatment of Loeb is applied to these eggs the centrifugal part cleaves better than the opposite pole even though this contains the nucleus. The heavy

materials in the egg thus favor cell division. Reverberi has made similar observations on unfertilized ascidian eggs which were separated into two parts by ultracentrifugation. After fertilization of the two fragments the heavy one, which originally lacked a nucleus, divides better than its partner. These results do not entirely contradict the findings of Beams and King on *Ascaris* which were mentioned earlier in this chapter, for, according to Beams and King, ultracentrifugation of the egg of this species stops cytoplasmic division without interfering with nuclear division. Thus, it may be the division of the cytoplasm that is especially dependent on these heavy granules, which occur only in the cytoplasm.

The importance of the finer structure of the egg cytoplasm for cell division is shown again by the recent observations of Monné (1944), where using the polarization microscope he was able to distinguish two different phases in the cytoplasm. One was an "enchylemma" fluid in which are suspended the yolk and mitochondria and the other a "structural phase" consisting of elongated fibrils containing chromidia rich in ribonucleic acid. The orientation of these fibrils may determine the position of the spindle during mitosis and the rate of segmentation may depend essentially on the relative proportion of the "enchylemma" and structural phases in various places in the cytoplasm.

One of the most fruitful problems of the future will be to work out the precise nature of the relations existing between carcinogenic agents, nucleoprotein granules, the —SH groups, and mitosis. Cancer research workers, as well as cellular physiologists, should find such studies profitable.

## 5. Conclusions

The essential points of this chapter will now be briefly summarized. The question of whether variations in respiratory metabolism accompany mitosis remains controversial, although the consensus of results favors the existence of metabolic changes during cell division. But it must be realized that these changes are quantitatively of little importance and, besides, the fact that segmentation often proceeds in a normal fashion under anaerobic conditions leads us to believe that variations in oxygen consumption during mitosis are only of secondary importance. Perhaps they are indications of rhythmic anaerobic reactions coupled with respiration. We

have only vague evidence for the support of this hypothesis, the chief point of which is the rhythmic production of acids resulting from carbohydrate metabolism (Runnström). It appears that metabolic variations serve solely to furnish the energy necessary for the work done by the cell at the time of division. There is nothing to show that the quantity of this work would be considerable (Rapkine, 1929).

The reasons why cells begin to divide remain completely unknown and none of the explanations proposed thus far are adequate. The action of "mitogenetic rays," in particular, tends to be discarded more and more, since a large number of recent works are not favorable to this hypothesis.

At the moment, the interpretation best adapted to the morphological and chemical changes which accompany development appears to be Rapkine's hypothesis of a reversible denaturation of the proteins during cell division. This fits in with the known facts concerning the localization of —SH groups and their beneficial effect on mitosis and also accounts for the fact that blocking of these groups stops cell division which recovers on the addition of sulfhydryl compounds. In addition, the hypothesis of Rapkine has the advantage of accounting for the structure of the spindle and its birefringence, and for the changes in viscosity which are characteristic of cell division. Finally, it contributes to an explanation of the migration of the chromosomes toward the poles if one admits that the protein spindle fiber returns to the globular state during anaphase and that it pulls the attached chromosomes. This concept is strictly analogous with prevailing ideas concerning muscular contraction which have a solid foundation, even to the reversible denaturation of the proteins and the profound modification of the structure of the proteins. The examination of the X-ray diffraction pattern of muscle (Astbury) demonstrates that the polypeptide chains tend to coil when muscle contracts and to uncoil and elongate during relaxation.

The fitting of these facts into the picture of the cell in mitosis seems warranted, but it will be proved so only when an analysis of X-ray diffraction pattern of the proteins of the spindle has been carried out.

The concept of Rapkine has the further advantage of allowing us to grasp the connection between the denaturation of proteins and metabolism. The changes in the concentration of —SH groups of proteins has, as we have already seen, an effect on the glutathione

concentration in the cell or, more precisely, an effect on the equilibrium between the oxidized and reduced forms of this tripeptide. But we know that the function of several enzymes depends directly on this equilibrium, notably in the case of proteases, succinic dehydrogenase, and triosephosphate dehydrogenase. For this latter enzyme, which is involved in an essential role in glycolysis, Rapkine has clearly demonstrated (1938) that oxidized glutathione inhibits it, while reduced glutathione on the contrary activates it. Thus it is easy to see why several investigators, notably Rapkine, and Pomerat and his collaborators, have foreseen the relation between mitosis and carbohydrate metabolism.

In this regard we see that one can make a new and fruitful comparison with muscle, since we have known for a long time that the metabolism of this tissue principally involves the carbohydrates. The more recent of the remarkable investigations of Szent-Györgyi and his school have established that there is a direct relation between glycolysis and the contraction of the myosin molecule. The Hungarian workers have, in effect, demonstrated that the element responsible for muscular contraction is *actomyosin*, a complex formed by two proteins, actin and myosin. Muscular contraction becomes a matter of combining actomyosin with magnesium and adenosine triphosphate, the latter acting as coenzyme for the phosphorylations accompanying glycolysis. The reaction which furnishes the energy necessary for contraction is the hydrolysis of adenosine triphosphate, which loses two molecules of phosphate; the enzyme producing this hydrolysis is myosin itself. It is a curious fact that this enzyme functions only when the complex actomyosin is found in the contracted state and it is thus the contraction of the molecules that brings into play the hydrolysis of adenosine triphosphate. It will evidently be of great interest to see if these ideas can be applied to the cell in mitosis and there should be no difficulty in determining if the amounts of adenosinetriphosphatase and adenosine triphosphate change during mitosis.

The hypothesis of Rapkine thus throws light on a number of obscure points and is based on certain facts. However, it would be dangerous and exaggerated to consider it as demonstrated and one can only judge it when it has withstood the test of experiment.

There is another peculiarity of mitosis which has still to be explained: the contraction of the chromosomes after prophase. We know that the chromosomes contain thymonucleic acid. Astbury

and Bell have studied the X-ray diffraction pattern of the fibers of this acid, which appears when the fiber is stretched as a column of flattened nucleotides which unite with a rod in the shape of a comb. The distance between the nucleotides is 3.34 Å—the interval separating the lateral chains in an extended polypeptide chain. As a result, the thymonucleic acid fits perfectly into a fibrous protein such as clupeine. If one brings it into reaction with a globular protein like edestin, the latter opens up and unfolds and becomes fibrous. The thymonucleic acid can thus impose its own inherent structure on proteins with which it reacts.

Astbury and Bell have noted that these properties of thymonucleic acid account for modifications which are undergone by the chromosomes during mitosis. If the distance between the nucleotides diminishes, the proteins will become globular and the chromosome will shrink. The dimensions of the chromosome will then be controlled by the interval between the nucleotides. One can imagine that these chromosomal changes favor an active migration of these structures toward the poles. It is, in any case, interesting to note that traces of  $\text{Ca}^{++}$  ions stimulate an extensive contraction of thymonucleohistone fibers (Jeener) and that this ion exerts the same effects on the chromosomes of the isolated germinal vesicle of the frog's egg (Duryee). It may be then that calcium is responsible for the shrinkage of the chromosomes at metaphase by acting directly on the configuration of the molecules of a nucleohistone.

The explanations which we have just been proposed tend toward a "mechanical" theory of mitosis. This is not to say that the electric charges of the proteins do not play a causal role which we can hardly yet evaluate. The fact that the nucleoproteins are "dipole" molecules permits the explanation of their behavior in certain cases, especially the pairing of the chromosomes during mitosis. This question is the subject of an interesting essay by Friedrich-Freksa, which we recommend to the reader who desires to go further into the problem. However, we must add that other hypotheses, such as that of Fabergé, for example, based on entirely different physical considerations, have been invoked to explain the pairing of the chromosomes.

Let us end by noting that our understanding of cell division has been extended largely due to recent biochemical research. One feels justified in believing that the analysis of the phenomenon of mitosis by methods which have given such brilliant results for the pure proteins and for muscle will shortly be extended along far-reaching lines.





## CHAPTER VI

# Synthesis, Localization, and Physiological Role of the Nucleic Acids

### A. SYNTHESIS OF THYMONUCLEIC ACID DURING EMBRYONIC DEVELOPMENT

#### 1. General Considerations

One of the most conspicuous results of cleavage is the increase in the number of nuclei. These cell divisions continue during the later stages of development, which are thus necessarily accompanied by the synthesis of nuclear constituents, especially of chromatin. Since this latter substance contains a high proportion of thymonucleic acid, it is reasonable to suppose a synthesis of this acid in the course of development.

These facts early attracted the attention of Boveri, R. Hertwig, Loeb, *et al.*, and Godlewski undertook, in 1908, to express them quantitatively by measuring the volume of nuclei and their number during the development of the sea urchin egg. The total volume of all the nuclei increased rapidly during cleavage and this growth was most rapid up to the 64-blastomere stage, after which it was much slower. Thus, cleavage modifies profoundly the nucleoplasmic ratio suggested by R. Hertwig, changing the values of the proportion  $\frac{\text{volume of nucleus}}{\text{volume of cytoplasm}}$  from  $1/550$  in the unfertilized egg to  $1/6$  in the blastula.

The investigations of Godlewski have been confirmed by J. Bury and by R. Erdmann who were careful to measure the volume of the chromosomes instead of the total nuclear volume, since this latter may contain a variable amount of nuclear sap in the different stages of development. They found during cleavage a decrease in the size

of the chromosomes, indicating that the synthesis of chromatin was not as rapid as Godlewski thought. Precise measurement of the volume of the very small chromosomes of the sea urchin is, however, quite difficult. Conklin, working on *Fulgur carica*, observed only a slow synthesis of nuclear substance during cleavage, and he stressed, quite appropriately, the fact that the amount of nuclear sap is very important. Since the nuclear sap does not necessarily contain nucleic acid, one can hardly make convincing deductions concerning the synthesis of this constituent using such methods.

This objection has been considered by Le Breton and Schaeffer who have severely criticized the methods of Godlewski. They point out, very justly, that the Polish worker measured only volumes, and from these alone one can draw no conclusions regarding masses. The fixation and dehydration before embedding often affects the relative volumes of the nucleus and the cytoplasm to a considerable degree, and it should be noted further that a small error in the measurement of the diameter of an object has an enormous effect on the determination of its volume. To these criticisms we must add that there is nothing to show that the concentration of nucleic acid in the nucleus is the same in different stages. We shall see, indeed, that it is quite the contrary.

In fairness to Godlewski it should be pointed out that he made no attempt to draw conclusions regarding the synthesis of thymonucleic acid and that he took the precaution of utilizing only very vague terms such as "nuclear substances" or "chromatin-like substances." His conclusions are thus legitimate if one does not consider these substances to be identical with thymonucleic acid, since the latter is only one of the nuclear constituents and can not be considered to correspond to the total "nuclear substance." As a matter of fact, it was Loeb who first translated morphological results into chemical terms and inferred the synthesis of nucleic acid during development. We shall see that, in spite of the objections to which this interpretation is open, Loeb's idea was fundamentally correct.

The following paragraphs summarize the American biologist's view of the synthesis of nucleic acid. The necessary phosphate is supposed to be derived from phospholipids, notably lecithin, which undergoes hydrolysis. The nitrogenous bases, purines and pyrimidines, are synthesized at the expense of unknown substances following an oxidative reaction and the fact that cleavage is not possible

under anaerobic conditions is thus explained. This last deduction is no longer valid since we have seen above that the eggs of Amphibia can divide perfectly well in the absence of oxygen and that there is no doubt that these eggs synthesize thymonucleic acid under anaerobic conditions.

Loeb was keenly interested in the observation of Godlewski that the rate of synthesis of nuclear substances was first slow, then accelerated rapidly between the 2-cell and 64-cell stages and finally slowed down again. Thus, if one draws a graph of the growth of the nuclei as a function of time, an S-shaped curve characteristic of monomolecular autocatalytic chemical reactions such as the autoxidation of linseed oil is obtained. In view of such a relationship Loeb felt justified in drawing the following conclusion: "The nucleus contains, or is in itself, an enzyme for the synthesis of nuclear substances or in other words this synthesis is an autocatalytic process." (*La fécondation chimique*, p. 330). The significance of S-shaped curves, so characteristic of growth phenomena, has been discussed at length by Needham (1931). Let us simply point out here that chemical and physical phenomena other than the one assumed by Loeb can give curves of this type. The foundations of Loeb's theory now appear to be very weak since they consist in morphological observations having a controversial biochemical interpretation and are not based on direct chemical analysis. Finally, they lean heavily on the S-shaped curve which is of rather doubtful significance.

It is to Masing (1910) that we owe the first attempt to verify Loeb's hypothesis by analyzing the amounts of nucleic acid phosphorus and purine nitrogen in the unfertilized sea urchin egg and in the fertilized egg at the blastula stage. His determinations showed *no change* in total nucleic acids in the egg during cleavage. Masing found 4.6 mg. of the purine nitrogen per 100 mg. dry weight whether the egg had one or 1000 nuclei.

These results of Masing's naturally reopened the whole problem again, and new work soon verified them. Shackell confirmed Masing's results, but his methods were criticized. Two students of Loeb, Robertson and Wasteney, attacked the problem by determining the protein phosphorus (which includes both nucleoprotein and phosphoproteins) and the lipid phosphorus. They observed an increase of the former at the expense of the latter, and, upholding Loeb's hypothesis, explained Masing's results by the fact that fertil-

ized eggs are always surrounded by supernumerary sperm which are rich in nucleic acid; these eggs will thus give a value that is too high, masking the synthesis of nucleic acid. However, as Masing showed in 1914, this criticism is not valid for unfertilized eggs and, moreover, the methods used by Robertson and Wasteneys were undoubtedly not as good as those employed by Masing. In addition, according to Fauré-Fremiet, the amount of protein phosphorus in the egg of *Ascaris* does not change during development. Because of such evidence, this author agreed with those opposing Loeb's ideas. It should be kept in mind, however, that, since the protein phosphorus includes both nucleoproteins and phosphoproteins, a synthesis of the first at the expense of the second is not revealed by lack of change in the value for the total protein phosphorus.

Godlewski (1918, 1925) attempted to correlate the chemical findings of Masing with his own results. It is well known that the oöcyte has a large nucleus filled with nuclear sap. If it were assumed that this germinal vesicle contains the material which gives rise to all the nuclei of the blastula, a series of migrations during development, beginning at maturation, would disperse the nuclear substance throughout the cytoplasm and, during the course of cleavage, it would be slowly taken up by the nuclei.

These two concepts, that of total *synthesis* of Loeb and that of *migration* of Godlewski have been opposing theories for many years. What are we to believe in light of recent investigations?

The question of the synthesis of nucleic acid was reinvestigated by J. and D. Needham in 1930 on a chemical basis, using the method of Plimmer and Scott for determination of nucleoprotein phosphorus. This method is complicated and open to objections but, nevertheless, it remains the best one available. The English investigators were careful to make many determinations on the eggs of various marine invertebrates (sea urchin, starfish, *Urechis*, crab and shrimp). They confirmed completely the findings of Masing, showing that the sea urchin egg does not synthesize nucleic acid during development in spite of the considerable increase in nuclear material visible microscopically. They came to the conclusion that the chromatin in the nuclei must draw on preformed nucleic acids dispersed in the cytoplasm of the unfertilized egg, in accordance with Godlewski's theory. The shrimp egg behaved like the sea urchin egg, while in the egg of the crab a slight synthesis of nucleic acid phosphorus was observed.

Finally, a more marked synthesis was observed in the starfish egg and especially in the egg of *Urechis*.

We have dealt so far only with the eggs of aquatic forms. In the eggs of terrestrial species the situation is not the same since the determinations at our disposal show that in the case of hen's egg (Plimmer and Scott, Fridericia), in the egg of the adder (Zinker-nagel), and in that of the silkworm (Tichomirov) there is a marked synthesis of nucleic acid. The determinations of nucleic acid phosphorus and purine nitrogen are in complete agreement in the case of these eggs. The behavior of the eggs of terrestrial forms as contrasted to that of marine invertebrate ova with reference to nucleic acid metabolism is shown in the table I taken from J. and D. Needham.

TABLE I

Species	Nucleic acid P present at the beginning of development in % of final amount
AQUATIC	
Sea urchin .....	100.0
Shrimp .....	100.0
Crab .....	77.9
Starfish .....	61.0
<i>Urechis</i> .....	34.6
TERRESTRIAL	
Silkworm .....	9.5
Hen .....	7.0

Considering these results, the Needhams believed that the eggs of the terrestrial animals studied synthesize their nucleic acids, as Loeb thought, while in the eggs of aquatic forms, preformed substances undergo a reorganization to form chromatin during cleavage. They recalled the interesting fact that the eggs of terrestrial forms excrete uric acid as an end-product of protein metabolism, while those of aquatic forms excrete ammonia or urea (see J. Needham 1931). It is tempting to compare the two phenomena, since uric acid is a product of the oxidation of the purines present in nucleic acids.

This concept, arrived at for different reasons by Godlewski and the English biochemists, is, however, not consistent with certain

facts that must be considered and which Brachet pointed out in 1931 and 1933. Numerous and convincing cytochemical findings have established that only traces of thymonucleic acid are found in the germinal vesicle and none at all in the cytoplasm. Since it has been shown in Chapter III that the Feulgen reaction is given exclusively by the chromosomes and is always negative in the cytoplasm, this fact and the fact that the egg, containing only traces of thymonucleic acid restricted to the chromatic regions, contains relatively large amounts of nucleic acid phosphorus and purines, are obviously contradictory. The one possible solution to this impasse is that there must be another nucleic acid in the egg, in addition to thymonucleic acid, which does not give the Feulgen reaction. Furthermore, since the total amount of nucleic acid does not increase during development in the sea urchin egg, there must necessarily be a conversion of this unknown nucleic acid, at least in part to thymonucleic acid. Thus, there will occur what might be called a *partial synthesis* during development. When one speculates on the probable nature of the nucleic acid that is converted into thymonucleic acid, one immediately thinks of ribonucleic acid. The idea that this substance might be present in the cytoplasm of the sea urchin egg seemed somewhat revolutionary at the time when it was suggested because it was then thought that ribonucleic acid was to be found only in plants, a theory now known to be completely incorrect.

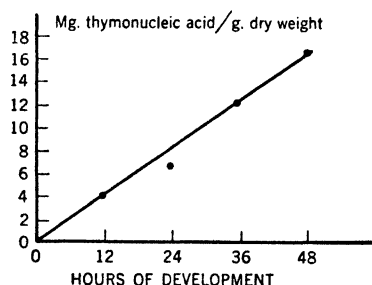
## 2. Partial Synthesis of Thymonucleic Acid

Brachet attempted to prove the hypothesis of partial synthesis by experimental studies (1931, 1933, 1936, 1937), the results of which showed, among other things, that the egg of the sea urchin *Paracentrotus lividus* gives only a faint Feulgen reaction sharply localized in the region of the nucleus. During cleavage the chromosomes show an intense reaction although the resting nucleus gives hardly any color at all. As cell division progresses the color increases even during interkinesis. The resting nuclei of the gastrula and of the pluteus give a very strong Feulgen reaction so that it must be concluded that the amount of thymonucleic acid in the nuclei increases progressively with development. It is evident that under these conditions the amount of nucleic acid in the nuclei can not be deduced from measurements of nuclear volume, since large nuclei are relatively poorer in nucleic acid than small ones. It is

further clear that there must be a considerable synthesis of thymonucleic acid during development.

These conclusions have been confirmed by several investigators for the eggs and embryos of various species. Voss has demonstrated in the *Axolotl* that during development, the nuclei of the morula give only a faint Feulgen reaction which becomes more intense with further cell division and that the color intensifies suddenly at the blastula stage. This is the time when the synthesis of thymonucleic acid becomes really important. The same holds true for *Triton* (Schönmann, Barigozzi) and for *Limnaea* (Raven); Stefanelli has recently studied the egg of *Rhabditis pellio* where the total nuclear volume and the nucleoplasmic ratio remains constant up to 256 blastomeres, but where the intensity of the Feulgen reaction increases continually. Therefore, in the egg of this nematode a synthesis of thymonucleic acid takes place without the increase of "nuclear substances" postulated by Godlewski.

Fig. 42. Synthesis of thymonucleic acid in sea urchin egg (J. Brachet).



The histochemical reaction of Feulgen, however, is not a quantitative technique and it is necessary to confirm the results gotten by its use by direct determinations of thymonucleic acid. This can be done by the colorimetric method of Dische, which allows us to establish definitely that there is actually a synthesis of thymonucleic acid during development, as shown in Figure 42 and in the following table:

Development, hours	Thymonucleic acid, mg.
0 .....	Trace (< 0.1 mg.)
12 .....	3.9
24 .....	6.7
36 .....	12.2
48 .....	16.4



The curve obtained is almost linear and does not agree with Loeb's hypothesis of a monomolecular autocatalytic reaction.

Similar results have been gotten, using the same method for the eggs and embryos of the frog, of an ascidian, of *Chaetopterus*, and of the trout (van der Ghinst). The absence of measurable quantities of thymonucleic acid in unfertilized eggs and the subsequent synthesis of this substance are, then, generally established facts. Brachet was also able to confirm the results of Masing and of J. and D. Needham in the sea urchin egg. Here are the figures obtained:

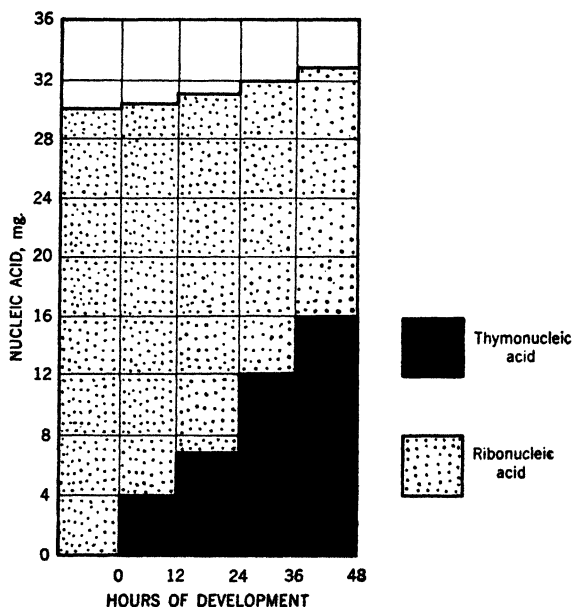


Fig. 43. Changes in the two types of nucleic acids in sea urchin egg (J. Brachet).

Nucleic acid P	{ unfertilized or fertilized eggs: 3.1 mg./g. dry wt. 40 hr. pluteus: 3.2 mg./g. dry wt.
Nucleic acid purines	{ unfertilized or fertilized eggs: 1.95 mg./g. dry wt. 40 hr. pluteus: 2.10 mg./g. dry wt.

It should be recognized, however, that the methods used for the determination of these two components are not completely satisfactory and that it is desirable that the question be re-examined when the techniques are refined. It seems however, that, beyond a doubt,

a *total synthesis* of thymonucleic acid corresponding to about 1.5 mg. of nucleic acid phosphorus or purine nitrogen should be excluded. If such a synthesis does occur it must be very slight and it is evident that the greater part, if not all, of the thymonucleic acid which forms during development has its origin in some other nucleic acid.

That this precursor of thymonucleic acid is ribonucleic acid appears to be well established by determinations of the amount of pentose in the egg, since this decreases with development as thymonucleic acid is synthesized, as shown in Figure 43.

Some older observations which have been neglected support the idea that egg cells contain ribonucleic acid. Levene and Mandel (1906) isolated from the eggs of the cod an acid similar to yeast nucleic acid. This substance was also found by Calvery in the chick embryo in which it occurs along with thymonucleic acid, and in which total synthesis occurs, there being a simultaneous increase in the amount of both thymo- and ribonucleic acids. In addition, Henze found pentoses and purines in abundance in the octopus egg, while Jenkinson observed these two components of ribonucleic acid to be present in the oöcytes of the frog. These investigations could not be interpreted correctly at the time of their publication because the two types of nucleic acid were not then well known.

The hypothesis of "partial synthesis" would seem, then, to be firmly grounded and explains the facts known at present. However, it has been criticized by G. Schmidt and by Blanchard, the former of whom, while confirming the fact that the sea urchin egg is rich in purines bound to nucleic acid, claims that the isolated nucleoproteins of the egg give the Feulgen reaction. If this were true, it would be necessary to invoke the concept of migration and admit the presence of a reserve of thymonucleic acid in the unfertilized egg. In reality, however, these nucleoproteins do not give a true Feulgen reaction but, rather, a plasmal reaction due to phospholipids, the color obtained having nothing to do with thymonucleic acid, as Brachet has shown (1936). Schmidt has, in a personal communication, agreed with this conclusion, for he has obtained the same color reaction in the yolk of the hen's egg which contains only traces of nucleic acid.

Turning to the work of Blanchard, who isolated from unfertilized sea urchin eggs (*Arbacia*) thymonucleic and ribonucleic acid in equally small amounts, we find that he concluded that Brachet's hy-

pothesis was not applicable to *Arbacia*. It should be noted, however, that Blanchard worked on kilograms of eggs and that it is not easy, under such conditions, to avoid contamination with fragments of the ovary which are full of nuclei rich in thymonucleic acid. The polar bodies containing Feulgen staining chromatin constitute another source of error. Finally, assuming that the thymonucleic acid isolated by Blanchard came from the eggs only, it would still be only 10% of the amount present in the pluteus. Thus, the observations of the American investigator do not constitute real evidence for a denial of a thymonucleic acid synthesis during development. On the contrary, the low yield obtained suggests that such a synthesis is probable. The present writer has confirmed the fact that one can isolate only small quantities of ribonucleic acid from unfertilized eggs. It seems probable, moreover, that methods used for extraction of yeast nucleic acid are not applicable to the egg, and Blanchard himself says that the two acids are not the same. The objections of this author are, therefore, not pertinent, since there is little likelihood that *Arbacia* behaves differently from *Paracentrotus*. It is none the less true that the ribonucleoproteins of the sea urchin egg have different characteristics from those of yeast and a thorough chemical study of the former remains to be done.

Summarizing the above facts, it is seen that unfertilized eggs never contain more than traces of thymonucleic acid and developing eggs synthesize large quantities of it. In the sea urchin egg this synthesis is linear with respect to time during segmentation, the plateau described by Godlewski and interpreted by Loeb as evidence of a monomolecular autocatalytic reaction not showing up when the thymonucleic acid is actually measured and therefore, only being connected with total nuclear volume. This discrepancy arises from the fact that the amount of thymonucleic acid in each nucleus increases during segmentation.

The purines of the sea urchin egg derive from two sources. These are, first, free mononucleotides, especially adenosine triphosphate (Örström, Zielinski, Lindberg) and, second, ribonucleotides combined with proteins, doubtless in the form of ribonucleic acids. It is the latter which take part in the liberation of ammonia during fertilization (Örström) and, according to all indications, they also give rise to the thymonucleic acid of the nuclei.

Some recent, unpublished observations of the writer confirm

this point of view. When the sea urchin egg is fixed, sectioned and stained with toluidine blue, the cytoplasm takes up much of the stain, but this affinity is lost in treating the sections with ribonuclease, indicating that the stain combined with a ribonucleic acid. The cytoplasm strongly absorbs ultraviolet at 2537 Å just like nucleic

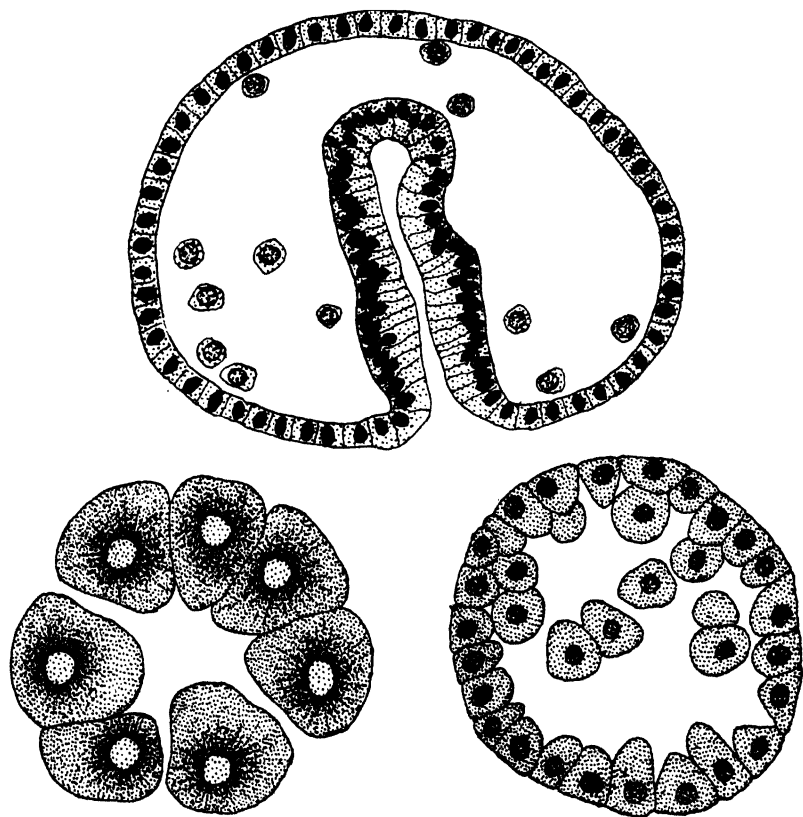


Fig. 44. Distribution of thymo- and ribonucleic acids in morula, blastula, and gastrula (J. Brachet).

acids (Harvey and Lavin) and in the ultracentrifuged egg, Harvey and Lavin have determined that the hyaloplasm absorbs ultraviolet more intensely than the yolk or the mitochondria. This conclusion sees confirmation by the observation of Runnström and Monné showing that the chromidia scattered throughout the hyaloplasm absorb

pyronin while the mitochondria do not become colored. During cleavage, the spindles and the asters in dividing cells and the cytoplasm adjacent to resting nuclei stain still more intensely with toluidine blue, giving evidence that these regions of the cell are exceptionally rich in ribonucleic acid. As development progresses, the affinity of the nuclei for the basic dye increases because of the increasing content of thymonucleic acid and correlated with this, one observes a decrease in the stainability of the cytoplasm as the concentration of ribonucleic acid decreases. Thus, cytochemical examination corroborates completely the results of chemical analysis and further evidence is adduced for the hypothesis that development is accompanied by a synthesis of thymonucleic acid in the nuclei with a parallel decrease in the amount of ribonucleic acid in the cytoplasm. Figure 44 illustrates, as well as representational facilities allow, these conclusions. Let us add here that decrease in basophilia was previously observed by Schaxel in the sea urchin egg and by Henneguy (1882) in the eggs of fishes. These two authors correlated this decrease of basophilia with the synthesis of chromatin, supposing that the latter substance came from a basophilic cytoplasmic substance of unknown chemical composition.

Probably one would find a decrease in the pentose values, accompanying the decrease in basophilia of the cytoplasm of the eggs of other forms showing partial synthesis (*Ascaris*, crab, shrimp, etc.). It can only be said that there is a satisfactory parallel between the content of nucleic acid phosphorus and pentoses in the unfertilized eggs of this type. Brachet (1936) found the ratio  $\frac{\text{nucleic acid P}}{\text{furfural}}$

to have a value of 0.45 in the sea urchin, 0.43 in the crab, 0.44 in a mollusk, and 0.40 in the starfish, suggesting the presence of a ribonucleic acid in the cytoplasm of these eggs.

Although the hypothesis of partial synthesis rests on numerous lines of evidence it may be said to have been rigidly demonstrated only when an *in vitro* synthesis of thymonucleic acid is accomplished by adding ribonucleic acid to a purée of eggs. Repeated attempts made by Brachet to this end have proved inconclusive, since in many tests there appeared to be a slight synthesis of thymonucleic acid but in amounts too small to be analyzed properly. This difficulty is not surprising, since ability to synthesize thymonucleic acid is the unique role of the chromosomes, which is lost when these cellular structures are destroyed in the technique employed.

It should be emphasized that there is no proof that ribonucleic acid is transformed *directly* into thymonucleic acid. On the contrary, it is likely that ribonucleic acid first undergoes hydrolysis and the molecule is probably broken down into more soluble mononucleotides, which are able to penetrate the nucleus. The hydrolysis probably goes still further, but the intimate biochemical mechanism of the synthesis of thymonucleic acid so far escapes us entirely. Nevertheless, it should merit an intense study.

### 3. Total Synthesis of Thymonucleic Acid

In the type of embryogenesis characterized by total synthesis of thymonucleic acid the unfertilized eggs contain only traces of nucleic acid phosphorus, purines, and pentoses. Development is accompanied by a simultaneous increase of the two types of nucleic acid. Actually, Mendel and Leavenworth claimed that synthesis of pentoses occurs during the development of the eggs of the hen and of the duck, and we have seen that Calvery isolated thymo- and ribonucleic acids from chick embryos, while on the contrary, the unfertilized or just fertilized hen's egg contains only small quantities of these acids. Let us also recall that the evidence for a nucleic acid synthesis during incubation comes from determinations of the phosphorus and of purines (Plimmer and Scott; Fridericia; Mendel and Leavenworth; etc.).

Recent work by Hevesy, Levi, and Rebbe has produced some enlightening details of the mechanism of this synthesis. Injecting the eggs with labelled phosphorus (radioactive P) in the inorganic state, the authors noted that it is later found, in part, in the nucleoproteins. Consequently, it may be supposed that the embryo synthesizes nucleic acids at the expense of inorganic phosphorus and it is not necessary to look to the lipid phosphorus of the phosphatides as a source of phosphorus as Loeb did. It may be noted in passing that the utilization of this same technique reveals a particularly rapid turnover of the phosphorus of nucleic acid in animal tumors (Kohman and Rusch; Brues, Jackson and Cohen) and in leukemia (Tuttle, Erf and Lawrence).

An important paper by Le Breton and Schaeffer is devoted to the problem of the metabolism of nucleic acids in the embryos of birds and mammals, and these authors deserve the credit for having posed the question of the nucleoplasmic ratio in a chemical way by proposing to express it in the following manner:

$$\frac{\text{purine N} \times 100}{\text{total N} - \text{purine N}}$$

This formula is, nevertheless, open to criticism for the reason that the purine nitrogen includes both types of nucleic acid, while only thymonucleic acid is localized exclusively in the nucleus. Finally, the nucleus is not composed of this acid only, and we do not know the chemical nature of the other constituents. Thus it appears preferable to adopt, as Brachet has suggested, a "chromatinoplasmic" chemical ratio which corresponds to the formula:

$$\frac{\text{thymonucleic acid N} \times 100}{\text{total N} - \text{thymonucleic acid N}}$$

The denominator in this ratio corresponds to the sum of the true cytoplasm and the yolk reserves which are included, this being a point which Le Breton and Schaeffer have justifiably emphasized.

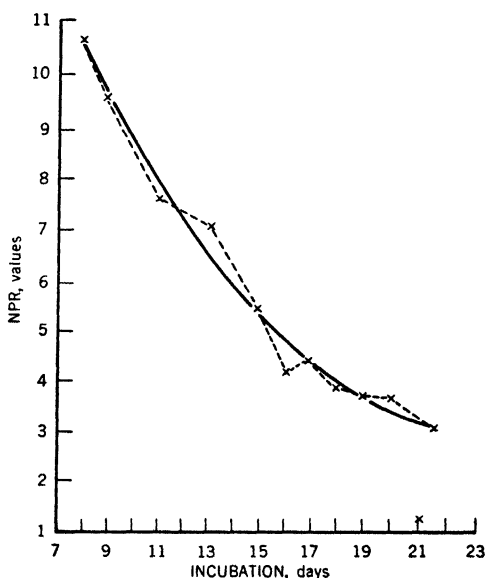


Fig. 45. Decline of nucleoplasmic ratio in chick embryo (Le Breton and Schaeffer).

The French physiologists noted a *progressive decrease* in the nucleoplasmic ratio during development of the hen's egg (Fig. 45) while, on the contrary, Brachet observed an increase in the egg of sea urchin. This disagreement is only apparent and is due to the

fact that the determinations were carried out on different stages of development, Brachet's results having been obtained from early stages of embryogenesis, while those of Le Breton and Schaeffer were gotten using advanced embryos. Actually, these authors admit that the decreasing phase which they discovered is preceded by a period during which the chemical nucleoplasmic ratio rises. Thus, we see that according to the stage of development the synthesis of purines is more or less rapid than that of total nitrogen. Hence, the embryo is the site of "disharmonies of growth" in its chemical composition, a point studied in detail by Teissier and by J. Needham (1931).

The results of Le Breton and Schaeffer have since been reinvestigated by Graff and Barth who drew analogous conclusions without, however, citing their predecessors. It would be well to interpolate here that Caspersson and Thorell established recently that the content of ribonucleic acid in the chick embryo decreased during the second phase of its development. This phenomenon would necessarily have an effect on the amount of purines in the embryo and is a good illustration of the fact that these derivatives can not serve as a chemical index of the quantity of chromatin. Thus, one could obtain a curve different from that published by Le Breton and Schaeffer if thymonucleic acid were substituted for total purines.

Among the reptiles, Zinkernagel studied the egg of the adder, which behaves like that of the hen. The amount of purines in this egg changes from 0.06 to 1.0 mg of purines in the course of development. The purine of the yolk is rather high and, contrary to what occurs in eggs of birds, tends to decrease somewhat in amount during development (0.38 to 0.34 mg of purine N). The chemical nucleoplasmic ratio,  $\frac{\text{purine N}}{\text{total N}}$ , increases between the fourth and the sixteenth days and decreases between the sixteenth and twenty-eighth days. This observation is thus in complete agreement with that of Le Breton and Schaeffer, but the same reservations should be made regarding the significance of the numerator as in the preceding paragraph.

Turning to the amphibians, the recent determinations of Graff and Barth (Fig. 46) show that cleavage is not accompanied by a synthesis of purines, but that this process begins later and soon becomes considerable. These investigators conclude that there may at first be a migration of nuclear substance as Godlewski supposed,



and then a total synthesis. They make no reference to the possible role of ribonucleic acid, which they apparently ignored, and suppose that thymonucleic acid in the cytoplasm passes into the nuclei. This conclusion contradicts the results obtained by use of Feulgen's technique (Voss, J. Brachet), which show that thymonucleic acid is synthesized even during segmentation. Since the nuclei are relatively poor in thymonucleic acid and few in number up to the blastula

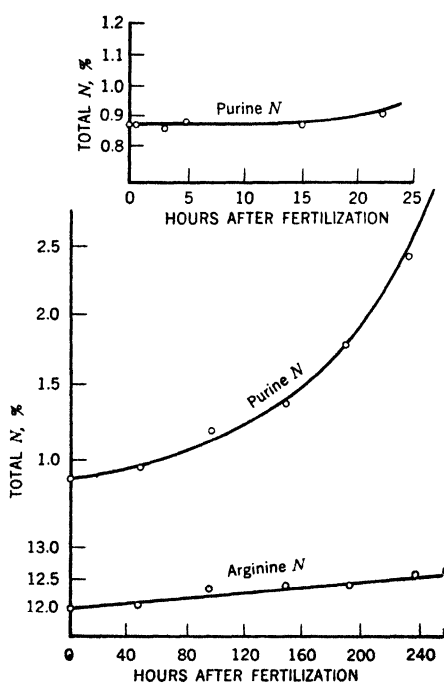


Fig. 46. Synthesis of purines and arginine during the development of frog egg (Graff and Barth).

stage it is conceivable that the synthesis of purines during cleavage can not be measured with precision. The work of Graff and Barth is interesting chiefly for establishing that the total synthesis of nucleic acids is more rapid during the period of differentiation than during cleavage, and this result is in agreement with the cytochemical findings to which we will return later.

The present writer established earlier (1937) that the egg of

the frog synthesizes about 4 mg of thymonucleic acid per thousand eggs from fertilization to hatching. He thought that this synthesis was paralleled by an equivalent decrease in the amount of pentoses and consequently placed the eggs of amphibia in the same class as those of the sea urchin with reference to nucleic acid metabolism. This conclusion turned out to be incorrect and has been modified (Brachet, 1941), since the decrease in pentose values is only apparent and results from the fact that the mucous jelly gives the furfural reaction upon hydrolysis; unfertilized eggs, of course, are provided with this jelly while the tadpoles have lost it. Actually a synthesis of ribonucleoproteins occurs during the development of amphibian eggs, which behave like those of the hen and of mammals and thus exhibit total synthesis. The only difference between the eggs of Amphibia and birds lies in the fact that the former already contain an appreciable quantity of ribonucleoproteins incorporated in the yolk before development begins.

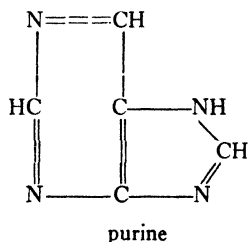
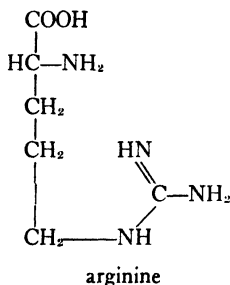
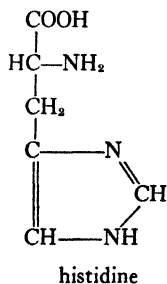
Finally, it has been long known that the eggs of the cod and herring contain large quantities of ribonucleic acid (Mandel and Levene; Levene and Mandel; Tschernorutzki, König and Grossfeld). Unfortunately, the ultimate fate of this ribonucleic acid has not been studied and thus we do not know if it is transformed into thymonucleic acid during the development of the egg. The case of the trout egg appears to be quite different, according to a study by van der Ghinst, who observed a total synthesis of the two nucleic acids much like what occurs in the hen's egg. He has determined the amounts of nucleic acid in the embryo and in the isolated yolk and found that synthesis takes place exclusively in the embryo. The yolk mass is devoid of ribo- and thymonucleic acids up to hatching of the young fry. It is certain in this case that the synthesis must occur at the expense of small molecules originating by the hydrolysis of the yolk. Cordier has definitely demonstrated that the vitelline syncytium through which the nutritive exchange between the embryo and the yolk takes place behaves like a dialyzing membrane. It may be that the hydrolysis of the yolk occurs at the surface of this syncytium, since it is known to contain enzymes (van der Ghinst).

Total synthesis is the rule in insect eggs as well (Tichomirov). This finding has been confirmed by Thomson and Bodine who have described a synthesis of "protein" phosphorus during the development of a grasshopper egg, which occurs only when growth is active and ceases during the period of diapause or when mitoses are stopped.

#### 4. Chemical Mechanism of the Synthesis of Nucleic Acids

One can only speculate on the chemical mechanisms of nucleic acid synthesis. The experiments of Hevesy and his collaborators show, as we have seen, that phosphorus introduced in an inorganic form is incorporated into the nucleic acid molecule. It is probable that the ribose comes from the catabolism of hexoses, since the work of Warburg and Christian and of Dickens has established that hexose-phosphate is transformed into ribosephosphoric acid by an oxidative decarboxylation (see Chapter V). The origin of the desoxyribose of the thymonucleic acid remains completely unknown and one can only imagine that it forms from the reduction of ribose but nothing certain is known regarding such a reaction.

As for the synthesis of the purines it is generally assumed, because of certain analogies in chemical constitution, that they are derived from histidine and arginine. As indicated by the formulas below, the structure of these amino acids recalls that of a purine.



The biochemical data furnished by a study of developing embryos unfortunately do not rigorously prove this hypothesis concerning the origin of purines. Schlenk's observations would seem to support this idea, since he found a decrease in arginine and histidine content during the development of the chick. The chick embryo of 6 to 7 days, incidentally, is characterized by an exceptionally high amount of diamino acids. These results were verified by Caspersson and Thorell, who pointed out that the absorption spectra of cytoplasmic proteins changes during incubation. The young embryo shows a maximum absorption at 2800 Å, which is characteristic of histones and results from the presence of large amounts of arginine and histidine. This maximum absorption disappears with time and the Swedish investigators correlate this de-

crease of histidine and arginine with synthesis of purines. These results, however, are not sufficiently quantitative to consider them as conclusively demonstrating this conversion. It should be noted, moreover, that the recent determinations by Graff and Barth obviate the participation of arginine in the synthesis of purines, for, as we have seen in Figure 43, development is accompanied by a simultaneous increase in the content of purines and arginine in the egg.

We owe to Edlbacher an interesting theory concerning the utilization of arginine in the synthesis of nucleoproteins. These

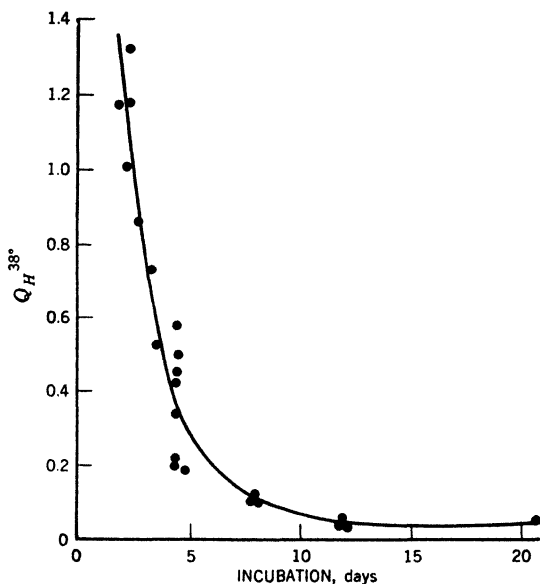


Fig. 47. Decrease in arginase activity of chick embryo during incubation (J. Brachet and J. Needham).

ideas have already been discussed in Chapter III and it is appropriate to discuss them again at this point. Considering that arginase takes part in the synthesis of arginine, which forms an important part of the basic proteins combining with nucleic acids (protamines, histones), Edlbacher based his idea on the abundance of arginase in cells in active growth, notably tumor cells and cells of mammalian embryos. This hypothesis has been supported by measurements made by J. Brachet and J. Needham; J. Needham, J. Brachet, and Brown; and Palladin and Rashba. All these investigators have

demonstrated that the amount of arginase in the chick embryo diminishes during development (Fig. 47); we know, of course, that the rate of growth also drops off during incubation.

However, we have not been able to detect arginase in the eggs of the sea urchin and of the frog during development, although they are the site of active synthesis of nucleoproteins. The large amounts of arginase in tumors is not a general condition as shown by Greenstein. Edlbacher's ideas thus do not seem to have general application, and it might well be asked whether the changes Brachet and Needham observed in the chick do not have some other significance. We know (see J. Needham 1931) that the young chick embryo excretes urea and finally eliminates uric acid. Since arginase plays a very important role in ureopoeisis (Krebs and Henseleit), it is not surprising to see the enzyme disappear at the time when the embryo stops the excretion of urea.

Furthermore, the recent research of Schoenheimer's school, using the heavy isotope of nitrogen, indicates that we must take a different view from that of Edlbacher. Indeed the American investigators have found that if one injects tagged nitrogen in the form of the ammonium salt into an animal, a fraction of this nitrogen is recovered in the purines and pyrimidines of the nucleic acids. The tagged nitrogen is not only present in the amino groups of these bases but also in the ring itself. These results strongly suggest that the animal is able to accomplish the total synthesis of the purine and pyrimidine nuclei starting with quaternary ammonia.

The problem of the synthesis of nucleic acids from the strictly biochemical point of view obviously requires long and patient study before a solution is found.

## 5. Conclusions

The essential fact established up to now is that the unfertilized egg contains only traces of thymonucleic acid and is not endowed with a reserve of this substance. In the course of later development thymonucleic acid is synthesized parallel with the increase in chromatin. The determination of thymonucleic acid permits us to follow quantitatively the multiplication of the chromosomes, but it does not allow us to draw any conclusions concerning the growth of the "nuclear substance," since the nucleus is not composed exclusively of thymonucleic acid; moreover, the amount of this substance in the

nucleus increases during development. The theory of migration of Godlewski can not thus be maintained in its simple form. It is certain that the nuclear sap of the germinal vesicle does not contain thymonucleic acid and that it can not be the source of the chromatin synthesized during cleavage. One can not exclude the possibility that the sap at first dispersed throughout the cytoplasm (at the time of germinal vesicle breakdown) is taken up again by the nuclei during cleavage. It would then form the nuclear sap of these nuclei without participating in the synthesis of chromatin.

The theory of Godlewski is connected with the hypothesis of "partial synthesis." We know that there can be no passage of thymonucleic acid from the cytoplasm into the nucleus during development since the egg does not contain a reserve of this acid. But the egg can accumulate in its cytoplasm ribonucleic acid which may be transformed into thymonucleic acid. Moreover, there is evidently a total synthesis of nucleic acids in the embryos of other species which conform to Loeb's theory.

It may be questioned whether these two different types of syntheses need be regarded as mutually exclusive. It would seem logical, on *prima facie* evidence, to associate total synthesis with terrestrial life and partial synthesis with aquatic environment (J. and D. Needham), but we now know that in the aquatic egg of the trout, in which the nitrogen metabolism is not uricotelic, there is a total synthesis of nucleic acids. Brachet has suggested a relation between the size and mode of cleavage of eggs and their synthesis of thymonucleic acid. Oligolecithal eggs with total cleavage would be the site of partial synthesis pursuing this scheme, while large eggs with partial cleavage and provided with a vitelline syncytium would be characterized by a total synthesis. This explanation is no longer completely valid, since we know that in the frog's egg, in which cleavage is total, there is a simultaneous synthesis of both types of nucleic acids. It seems, in reality, that an antithetical distinction between the two modes of synthesis is a gratuitous difficulty. As a matter of fact, there exist all intermediate types between the eggs of the sea urchin and the hen, as already shown by the determinations of J. and D. Needham (Table I, p. 193).

It may well be asked how a synthesis of the foregoing facts can be accomplished. To begin with, the cytochemical study of oögenesis reveals that the young oöcyte is rich in ribonucleic acid regardless of

the species under consideration. It is also a general phenomenon that during later development there is a decrease of this cytoplasmic ribonucleic acid. This decrease, however, is not of the same order of magnitude in different types of eggs. Small in the egg of sea urchin, where the yolk contains ribonucleic acid in abundance, it is much more marked in the frog egg, where the yolk contains less of this nucleic acid, and in the eggs of the hen and the trout, this increase is considerable, since their yolk does not contain ribonucleic acid at all. If, by centrifugation, we separate the deutoplasm from the hyaline cytoplasm, we see that the latter remains rich in ribonucleic acid whatever type of egg is used. The situation of the nucleic acids in the mature oöcyte or in the unfertilized egg presents a number of constant features: the presence of thymonucleic acid in small quantities in the nucleus only and the existence of ribonucleic acid in the hyaline cytoplasm. That which differs from one species to another is the quantity of yolk and its chemical nature. It appears that the greater the amount of yolk in the egg, the less the amount of ribonucleic acid in the deutoplasm.

What happens, then, during cleavage? We can envisage two hypotheses: either the decrease in the amount of ribonucleic acid in the egg of the sea urchin during its development is purely fortuitous and has nothing to do with the synthesis of thymonucleic acid, or, this same decrease takes place in the eggs of other species as well, and constitutes a general phenomenon but is masked in eggs with "total" synthesis by the simultaneous formation of ribonucleic acid.

The first alternative is less probable than the second one, by reason of the constancy of the amount of nucleoprotein phosphorus and purine nitrogen during the development of the unfertilized egg to the pluteus, a fact that has been confirmed by all authors who have studied the problem. The question is of sufficient importance to warrant re-examination with newer techniques as they are developed. Certainly, the fact that the utilization of ribonucleic acid is strictly equivalent to the synthesis of thymonucleic acid can hardly be a coincidence. Finally, cytochemical examinations, which should be carried out quantitatively using Caspersson's method, confirm the idea that the development of the sea urchin egg is accompanied by a decrease in the amount of ribonucleic acid and by a simultaneous synthesis of thymonucleic acid.

The second hypothesis assumes that even in the case of the eggs

of the trout and the hen thymonucleic acid derives from cytoplasmic ribonucleic acid but that a simultaneous synthesis of this latter substance occurs for other purposes, and that, the synthesis of ribonucleic acid being more rapid than its utilization during mitosis, the amount of pentoses in the egg will increase in the course of development. It is further supposed that in telolecithal eggs, for example, the soluble products of digestion of the yolk get to the embryo by means of the vitelline syncytium, and that in each cell of the blastodisc there would occur a synthesis of ribonucleic acid, a part of this acid being used in the formation of thymonucleic acid during mitosis. We will return to this second hypothesis later when some points necessary for a full discussion of it will be added. Its principal advantage appears to be the fact that it brings together into a coherent scheme the facts collected on eggs which differ widely, and accounts for the basic phenomenon of the synthesis of thymonucleic acid at the expense of ribonucleic acid, which substance can either pre-exist in the cytoplasm of the unfertilized egg, in the form of components or as a complete entity, or be synthesized *de novo* at the expense of the stored yolk.

Summing up, in the embryogenesis of various species of animals, ribonucleic acid is synthesized at different stages. In certain species this synthesis takes place during oögenesis, while in others it begins only after fertilization.

It is necessary to keep one point clearly in mind: that the nucleic acids, including thymonucleic acid, are not stable constituents of the cell. The research of Hevesy on the utilization of radioactive phosphorus by cellular nucleoproteins has established that these acids are in a state of continual flux. His method, which should be applied in a systematic fashion to eggs in the course of development, really proves that the phosphorus of nucleic acid is replaced constantly by fresh phosphorus, producing a continual turnover of these substances. This notion is new and important and the substance of the chromosomes, for example, appears now to be in a state of continual regeneration, having a metabolism the nature of which escapes us as yet but whose role, from the genetic point of view, must have profound significance. Some indication of the physiological role of phosphate turnover in thymonucleic acid is found in the investigations which show that this phenomenon is faster in animal cancers (Kohmann and Rusch; Jackson and Cohn; Tuttle, Erf and Lawrence)



and during regeneration of the liver (Brues, Tracy and Cohn). Thus it appears that the phosphate turnover may be, to some extent, coupled with the mitotic activity of cells. The fact that a direct parallel exists between the rate of turnover in the phosphate in thymonucleic acid and the amount of alkaline phosphatase of the nuclei (Brachet and Jeener) suggests that the reaction is controlled by this enzyme which is localized in the chromosomes and the nucleoli.

## **B. LOCALIZATION AND PHYSIOLOGICAL ROLE OF THE NUCLEIC ACIDS**

### **1. Localization in the Resting Cell**

It will be remembered that the cytochemical detection of thymonucleic acid was made possible about 20 years ago when the Feulgen reaction was introduced. We have already had occasion to examine in various connections the results of this method applied to developing eggs. The conclusions drawn are valid for all the cells of the organism and it may be considered as demonstrated that thymonucleic acid is localized exclusively in the region of the chromatin, at least in healthy cells.

The techniques used for the detection of ribonucleic acid are much more recent, although as early as 1913 Van Herwerden had observed that the basophilic granules (chromidia), which fill the cytoplasm of the sea urchin oöcyte, lose their staining ability after the action of a nuclease extracted from the spleen. Unfortunately, this preparation was a mixture of enzymes and, in addition, showed low activity so that the observations of Van Herwerden have not received the credit they deserve. We have seen that the sea urchin egg contains ribonucleic acid and it is logical to assume that this substance is in the cytoplasm, for if ribonucleic acid were a constituent of the nucleus we would necessarily expect it to be synthesized during development. But we know that just the contrary occurs, at least in the sea urchin egg. Furthermore, Jorpes has shown that the pentose content of testis tissue decreases during spermatogenesis, the mature sperm containing only traces of pentose. The almost total disappearance of pentoses in this case parallels the reduction in the amount of cytoplasm. All these facts led Brachet (1933) to believe that ribonucleic acid ought to be principally a cytoplasmic constituent probably localized in the region of the

chromidia. Several cytologists have noted a tendency for the disappearance of these chromidia during development of the sea urchin egg (Schaxel, J. Bury), and Schaxel suggested that they serve for the building of the nuclei during cleavage. The use of modern methods, particularly the utilization of purified ribonuclease, leaves no further doubt of the validity of this idea, and the properties of the basophilic material present in the cytoplasm of the sea urchin egg correspond well with those of a ribonucleoprotein (see Fig. 44).

The first direct demonstration of the presence of ribonucleic acid in the cytoplasm was made by Behrens. With the aid of a long and complicated method he succeeded in separating the nuclei from the cytoplasm by taking advantage of the difference in their specific gravities. However, Behrens studied only plant cells and his observations are limited to showing that, in the embryos of cereal grains, the cytoplasm contains ribonucleic acid while the nuclei contain thymonucleic acid (Feulgen, Behrens and Mahdihassan). Since these results did not concern animal cells, the general tendency—still prevalent—was to believe that animal cells contained only thymonucleic acid, and plant cells only ribonucleic acid. This distinction, both unfortunate and incorrect, between “animal” and “plant” nucleic acids is still met with too often in treatises on biochemistry.

The proof of the presence of ribonucleic acid in the cytoplasm of animal cells was furnished by Caspersson and Brachet. The conclusions drawn by Caspersson from his studies on the ultraviolet light absorption of microscopical preparations have been attacked recently by F. G. Fischer. The method used by Caspersson does not make a distinction between the two types of nucleic acid and his demonstration of ribonucleic acid was predicated on the presence of structures which absorb ultraviolet light in the same wave length as nucleic acids and which do not give the Feulgen reaction. Fischer objected that when thymonucleic acid is depolymerized by an enzyme, desoxyribonuclease, it ceases to give the Feulgen reaction, and it might well be that the cytoplasmic nucleotides demonstrated by Caspersson were those of depolymerized thymonucleic acid and not of ribonucleic acid. Brachet (1941, 1945) was able to show, however, that the criticisms of Fischer were not well-founded. The cytoplasmic nucleoproteins lose their basophilia after treatment with purified ribonuclease, which has no action on thymonucleic acid, and desoxyribonuclease does not affect the staining of these nucleopro-

teins although it causes the Feulgen reaction in the nucleus to disappear (the reaction becomes negative because the depolymerized thymonucleic acid is very soluble and not because its composition is modified in such a way as to be incapable of giving the Feulgen reaction). Finally, there is a perfect parallel between the basophilia of different organs and their pentose content.

The results obtained by Caspersson and by Brachet, working simultaneously and independently and using entirely different techniques, are in complete agreement. Caspersson and Schultz observed that the root tips of *Allium cepa* and the imaginal discs of *Drosophila* larvae have a high content of cytoplasmic ribonucleic acid. They naturally concluded from this that there was some relation between this substance and *growth capacity*. But Brachet (1940) showed, at the same time as Caspersson, that tissues in a state of proliferation are not alone in containing ribonucleic acid in large quantities. It was also observed in abundance in the cytoplasm of the secretory cells of the pancreas, in the Nissl bodies of nerve cells, in young oöcytes, etc. In addition it was found in the nucleolus, whose basophilia disappears after treatment with ribonuclease. In 1941, as a result of intensive study in both laboratories, the same conclusion was drawn by Caspersson, Landström-Hydén and Aquilonius, and J. Brachet: that there is a direct relation between the content of ribonucleic acid of a cell and its capacity for *synthesizing proteins*. When protein synthesis is active in a cell the basophilia of the cytoplasm and the nucleolus increases, as do the absorption in the ultraviolet, the pentose content, and the nucleic acid phosphorus. Now, organs physiologically very active, like the heart, striated muscle, kidney, lung, and the endocrine glands, are very low in ribonucleic acid, and their manufacture of protein is undoubtedly limited to the synthesis of their own proteins. In the case of the endocrine glands, incidentally, it is interesting to note that the stimulation of hormone secretion by injection of oestrone is accompanied by a marked increase in the ribonucleic acid content (Desclin, Herlant). Similarly, Dougherty and White have reported an increase in the amount of ribonucleic acid in lymphocytes after injection of the corticotropic pituitary hormone. On the contrary, cells that produce proteins in abundance are always strongly basophilic because of the large amount of ribonucleic acid which they contain, as for example, the cells of the pancreas, of the parotid

gland, of the gastric mucosa (especially in the region of the "principal" cells, which very probably secrete pepsin), of the intestine of vertebrates and invertebrates, and of the nurse cells in the insect ovary, etc. The plasmatocytes to which is attributed the secretion of the globulins of blood plasma are also characterized by a high content of ribonucleic acid (Zylberszac, van den Bergh; Bing, Fagraeus and Thorell). This acid is also found in abundance wherever there are numerous mitoses, notably in the basal layer of the skin, the crypts of Lieberkühn, and the hair follicles. It accumulates in the most active parts of the embryo (see later), and in regeneration buds after section in animals (H. Clément) and in plants. To our knowledge, the organ richest in ribonucleic acid is the silk gland of the silkworm, which has as its sole function the synthesis of a protein: silk. Finally, it is remarkable that microorganisms such as yeasts, bacteria (Graff and Barth), animal and plant viruses (Claude, MacFarlane *et al.*; Smadel *et al.*; Pollard; Bawden and Pirie; Stanley and Loring), where we know multiplication is rapid, are always very rich in nucleic acids.\* The volutin of yeast cells (Delaporte, J. Brachet, Caspersson and Brandt) and of trypanosomes (van den Berghe) is actually a ribonucleoprotein. Ribonucleoproteins are thus abundant in all cells which secrete proteins or synthesize their own proteins in large quantities during multiplication. This fundamental conclusion receives abundant confirmation from the analyses and cytochemical studies of Davidson and Waymouth; Levaditi and Veillet; Dempsey and Wislocki, *et al.*

It may be added here that the ribonucleic acid content of cells can sometimes be modified experimentally. We have already seen that the stimulation of the secretory activity of the hypophysis is accompanied by an increase in the ribonucleic acid content. An excessive stimulation of a gland by an electric current or by pilocarpine results, however, in a decrease in basophilia (Verne, Ries) and Caspersson, Landström-Hydén and Aquilonius have verified the finding that the amount of ribonucleic acid in the pancreas is lowered after intense stimulation with pilocarpine. In the liver, prolonged fasting results in a small decrease in the amount of ribonucleic acid (Kosterlitz; Davidson and Waymouth; Brachet, Jeener, Rosseel and Thonet; Opie; Opie and Lavin, *et al.*). Recently, Hydén has shown

\* The reader who desires more details on the subject of nucleic acids in microorganisms is referred to the recent review by A. Boivin.

that in nerve cells the synthesis of proteins which occurs with growth or upon electrical stimulation is accompanied by an increase in ribonucleic acid content. An increase in basophilia under these conditions was noted years ago (cf. Bayliss: *Principles of General Physiology*). Finally, the chromolysis following motor root section is accompanied by a diminished ribonucleic acid content of the cell, probably brought about by the hydrolysis of this acid into mononucleotides under the influence of ribonuclease present in the cytoplasm of neurones (Gersh and Bodian). Furthermore, it is well to note that chromolysis is not only accompanied by variations in the amount of ribonucleic acid in the neurones but also by interesting changes in the amount of acid phosphatase and cytochrome oxidase.

If the localization of ribonucleic acids in the cytoplasm is studied it is found that they form a part of the *ergastoplasm*, that is to say, basophilic granules with a lamellar structure present in cells having an intense secretory activity (Garnier and Bouin; Prenant). That the ergastoplasm functions in the formation of secretory granules or yolk platelets is a suggestion that has frequently been made. Furthermore, certain cytologists (see especially the article by A. Prenant) consider the ergastoplasm as a mass of mitochondria aggregated by fixation and in this case the mitochondria would be the morphological framework for the ribonucleic acid. At present there is not enough information to answer this question, one that merits thorough study. It is likely, however, that very different chemical entities are classified as mitochondria. The minute rods described by Heidenhain in the cells of the kidney certainly do not contain ribonucleic acid, while it is very probable that the mitochondria of intestinal cells do.

Another point which seems well established is that the nucleolus uniformly contains ribonucleic acid but in variable amounts. In general, there is a parallel between the amounts of ribonucleic acid in the cytoplasm and in the nucleolus, as noted by Caspersson. However, this rule does not hold absolutely since in developing amphibian embryos Brachet found that the endoderm cells (future digestive tract) show large and very basophilic nucleoli from gastrulation on, while the cytoplasm of these cells, on the contrary, contains only traces of ribonucleic acid at this stage. Ribonucleic acid does not appear in appreciable quantities in the cytoplasm until the digestive tract begins to show histological differentiation. Further-

more, in the notochord the cells lose their ribonucleic acid at the time of vacuolization while the nucleoli remain basophilic for a long time. Too, the cells of amphibian blastulae have a basophilic cytoplasm when the nucleoli are not yet basophilic. Finally, during oögenesis in certain insects and amphibia there occurs a decrease in basophilia of the cytoplasm accompanied by an increase in that of the nucleolus. Thus, the parallel between nucleolar and cytoplasmic basophilia should not be taken too literally, although, as indicated above, it is a general rule.

The writer's observations indicate that the *chromatin* contains a little ribonucleic acid along with greater quantities of thymonucleic acid. If microscopical preparations are stained with Unna's mixture (methyl green-pyronine), thymonucleic acid is observed to take up the methyl green while the ribonucleic acid shows an affinity for pyronine. The chromatin then takes on a blue tint and the cytoplasm is colored red, but after the action of ribonuclease the chromatin stains clear green while the cytoplasm and the nucleolus are colorless. These facts could not be demonstrated by Caspersson's method in its present form since it does not distinguish between the two nucleic acids. It would be interesting to know the effects of purified nucleases on the ultraviolet absorption of chromatin. In any case, Brachet has confirmed his results by pentose determinations made on nuclei isolated according to the method of Stoneburg. In the case of the cells of the frog's intestine 10% of the total nucleic acid is ribonucleic acid and the remainder is thymonucleic acid.

Some interesting results have been gotten from the study of the giant chromosomes of the salivary gland of *Chironomus*, which are made up of two distinct parts. The *euchromatin* consists of a series of discs rich in thymonucleic acid (corresponding probably to the genes) alternating with achromatic regions. This orderly structure is lost, however, in the *heterochromatin* which is denser and stains more intensely and which appears to play an important role in the formation of the nucleolus (Heitz, McClintock, Bauer). After staining with Unna's mixture, the discs of euchromatin take up methyl green almost exclusively, indicating that they are composed of thymonucleic acid, while, on the contrary, the heterochromatin takes on a violet tint which after the action of ribonuclease becomes green. It is thus only the heterochromatin which in this case con-

tains both nucleic acids. The presence of ribonucleic acid in heterochromatin is interesting by reason of its role in the formation of the nucleolus. The latter in *Chironomus*, as in other forms, is filled with ribonucleic acid.

In summary, chromatin contains thymonucleic acid and a little ribonucleic acid, the latter being found chiefly in the heterochromatin which gives rise to the nucleoli to which they are attached.

The nucleolus itself contains ribonucleic acid but no thymonucleic acid. The cytoplasm contains a variable amount of ribonucleoproteins, depending on its ability to synthesize proteins. These cytoplasmic ribonucleoproteins are localized in the region of the ergastoplasm.

## 2. Nucleic Acids in the Cell during Mitosis and the Chemical Composition of the Chromosomes

Cytologists have known for a long time that the staining of chromatin changes during cell division (see Heidenhain, for example). Similar observations have been made by E. Hammarsten, G. Hammarsten and T. Teorell; Kelley; and Luone, who noted that the affinity of the chromosomes for basic dyes like toluidine blue is greater than that of the resting nucleus. Kelley showed that this increased affinity for basic dyes is probably related to an increase in the amount of nucleic acid in the chromosomes.

Do modern cytochemical observations confirm this interpretation? The Feulgen reaction indicates that during cleavage of the egg the amount of thymonucleic acid in the nucleus changes radically.)  
 ✓The writer reported (1933, 1937) that the resting nuclei of the sea urchin egg or of the *Chaetopterus* egg during cleavage contain only traces of thymonucleic acid, although the chromosomes in metaphase give an intense Feulgen reaction.) Hence there are apparently rhythmic variations in the amount of thymonucleic acid in the nucleus.  
 ✓Actually, the reaction is nearly negative in the resting nucleus, becoming more intense during prophase, reaching a maximum at metaphase and shading off more and more in anaphase and especially at telophase. A similar series of events has been noted in amphibian eggs (Voss, Brachet), as well as in those of *Tubifex* [F. E. Lehmann], so they seem to have general significance, but it must be confessed that these rhythmic variations are less easily demonstrable at the end of cleavage. In the blastula, for example, the resting nucleus is

rich in thymonucleic acid and variations in the amount of this material during subsequent mitosis could only be established by quantitative methods. <sup>7</sup> Apropos of this subject, it is interesting to note that Caspersson (1936, 1939, 1940) has stated that the nucleic acid content of the nucleus changes during meiosis in the grasshopper.

✓ The use of methyl green-pyronine for staining, combined with the ribonuclease treatment, permits further analysis (Brachet, 1942). It has just been noted that thymonucleic acid selectively fixes methyl green while ribonucleic acid has a greater affinity for pyronine and that in the resting cell the two nucleic acids occur together in the chromatin, particularly so in the heterochromatin. ✓ Chromatin has, of course, a preponderance of thymonucleic acids. ✓ Finally, the nucleolus and the cytoplasm contain only ribonucleic acid. ✓ The changes that occur in these relationships during mitosis may now be considered.

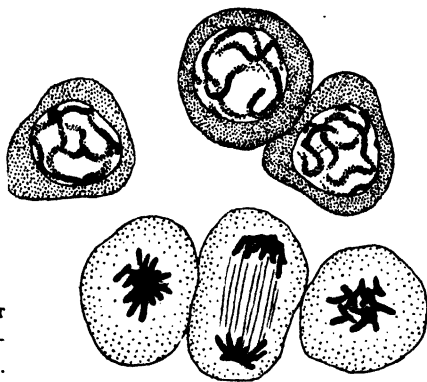


Fig. 48. Spermatocyte of grasshopper in the resting state and in mitosis: distribution of ribonucleic acid (J. Brachet).

✓ At the beginning of the mitotic cycle the affinity of the cytoplasm for pyronine decreases and the cytoplasmic structure changes visibly. In the resting cell ribonucleic acid is localized in large masses of ergastoplasm, but during prophase it tends to be dispersed. In the dividing cell the cytoplasm appears much more homogeneous and after fixation it is filled with small granules dispersed throughout the cell. In addition one often observes the presence of ribonucleic acid in the achromatic figure (spindle, asters). This detail, very striking in cleaving eggs, is less evident in ordinary cells. The chromatin at this time takes up pyronine more intensely and like heterochromatin, stains a bluish violet, probably containing a higher



proportion of ribonucleic acid.\* The difference between resting cells and dividing cells is well demonstrated in the cleaving eggs of urodeles and during spermatogenesis in the grasshopper (Fig. 48) where one sees clearly the decrease of cytoplasmic basophilia accompanied by a change in the staining reaction of the chromosomes at the time of the meiotic mitoses.† In this particular case the achromatic figure stains only slightly with pyronine. These same variations in the affinity of the chromosomes and the cytoplasm for methyl green and pyronine have been found in other materials by Matthey.‡

It might be thought that these variations in staining have no chemical significance and that the more marked affinity of the chromosomes for pyronine is caused by morphological changes.\* Von Möllendorf, in particular, considered histological staining reactions to be simple absorption phenomena controlled by the "density" of the structures being studied (*Strukturdichte*).¶ But the use of ribonuclease excludes such an hypothesis, since after the action of this enzyme the cytoplasm and the achromatic figure lose all affinity for pyronine as do the chromosomes which are stained a clear green like the resting nucleus, although the "density" of their structure has not been modified. Thus it seems that the chromosomes during their formation and up to metaphase are the site of greater ribonucleic acid formation than is the resting chromatin. Little by little this excess ribonucleic acid is lost during anaphase and telophase.‡ Ultraviolet absorption measurements of chromosomes and of resting nuclei before and after the action of ribonuclease would settle the question on a quantitative basis.‡

The variations in the concentration and localization of nucleic acids brought about by mitosis can be represented in the following way. At prophase the nucleolus disappears and the subsequent fate of its ribonucleic acid is not known. At the same time the amount of ribonucleic acid in the chromosomes increases—perhaps at the expense of the nucleolus—the basophilia of the cytoplasm decreases, and the ergastoplasm is resolved into small granules. At metaphase, part of the ribonucleic acid goes into the achromatic figure, but the remainder of the cytoplasm contains very little of it. The amount of ribonucleic acid, and probably of thymonucleic acid, continues to increase in the chromosomes. Telophase is seen to produce a progressive return to the initial state; the cytoplasm becomes filled with ribonucleic acid, the ergastoplasm reforms, and the achromatic figure fades while the nucleolus reappears in contact with the heterochromatin. Finally, the ribonucleic acid in the chro-

matin is reduced in amount. These cytochemical observations agree perfectly with those made on the synthesis of thymonucleic acid during the development of the sea urchin egg and suggest a participation of ribonucleic acid in the synthesis of thymonucleic acid. Figure

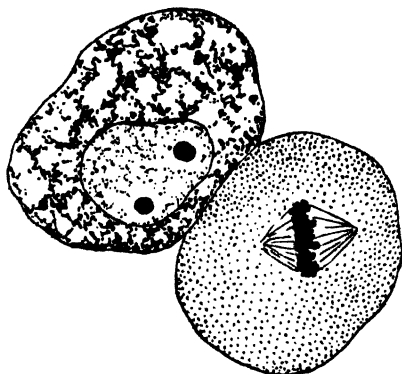
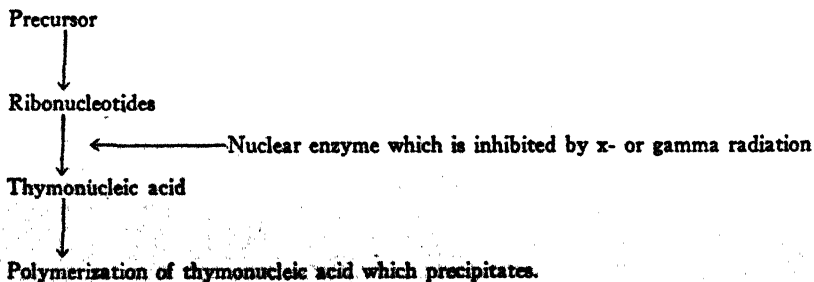


Fig. 49. Distribution of ribonucleic acid in the resting cell and in mitosis (J. Brachet).

49 illustrates the facts just described. It should be emphasized that too much value should not be attributed to it since differences certainly exist between different types of cells.

↓ The idea that thymonucleic acid is synthesized at the expense of ribonucleic acid in all cells in the mitotic state and not only in the egg of the sea urchin finds additional support in the recent observations of Mitchell. This investigator, using Caspersson's method, studied the nucleic acid content of cancer cells after irradiation with x-rays or gamma radiations. He observed an accumulation of ribonucleic acid in the cells where division had been blocked and concluded that radiations inhibit the transformation of ribonucleic acid into thymonucleic acid. The scheme below for the synthesis of thymonucleic acid fits in perfectly with the results Brachet obtained earlier in eggs.



↓ Mitchell's concept has received support from some observations by Stowell who, using a spectrophotometric method, showed that irradiation with x-rays stops the synthesis of thymonucleic acid.

↓ Note also that certain authors, Painter, in particular, think that the reverse transformation of thymonucleic acid into ribonucleic acid is equally possible. The fact that the nucleolus, rich in ribonucleic acid, appears in direct contact with the heterochromatin which contains large amounts of thymonucleic acid, may signify, according to Painter, that thymonucleic acid is transformed into ribonucleic acid in the region of heterochromatin. Painter has also put forth the interesting hypothesis that the chromosomes eliminated during chromatin diminution in *Ascaris* undergo a transformation from thymonucleic acid into ribonucleic acid; this would permit the continuation of mitotic activity and would stimulate morphogenesis. ↓ In this manner the fact that the cells of the germ line, where elimination of chromatin does not occur, remain inert for a considerable time is explained. ↓

Some observations in conformation with the hypothesis of a possible transformation of thymonucleic acid into ribonucleic acid have been made by other authors: thus the nuclei of pycnotic cells lose their affinity for methyl green, while they stain more and more deeply with pyronine. This latter staining disappears under the action of ribonuclease in the case of the thymus according to Dustin's observations. ✕ The same phenomena can be observed in the degenerating spermatocytes in the grasshopper (unpublished observations by Brachet). Finally, Pollister and Lavin, using the ultraviolet microscope, studied the interesting case of the "apyrene" spermatozoa in which the nucleus completely degenerates and simultaneously the cytoplasm becomes richer in ribonucleic acid.

↑ However tempting these correlations may be, we must not forget that they are as yet hypothetical in nature and that they will remain dubious until we can demonstrate experimentally the possibility of a transformation of one nucleic acid into the other. ✕

✓ Let us now see what results Caspersson and his collaborators have obtained with their method in regard to the changes in nucleoproteins during mitosis. Since their technique actually does not allow them to follow the variations in the two types of nucleic acids during mitosis, the Swedish investigators have nothing to say about the observations which have just been summarized. However, the

ultraviolet absorption technique has the great advantage of giving information on the nature of the *proteins* combined with nucleic acids, a circumstance that has resulted in the acquisition of some extremely important facts.

The first point made by Caspersson and Schultz and by Schultz and Caspersson is that a direct relationship exists between the two nucleic acids. We have already seen that the introduction of a Y chromosome into a female *drosophila* (XX) results in an increase in the concentration of cytoplasmic ribonucleoproteins. The heterochromosomes also modify the chemical constitution of the nucleoli in the salivary glands; the absorption spectra of the latter structures differ in male and female as shown very clearly in Figure 50.

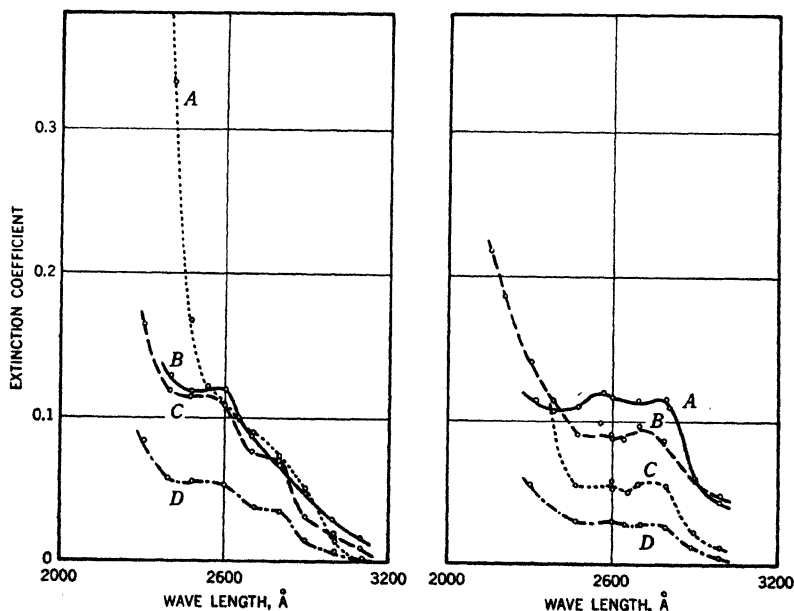


Fig. 50. Absorption spectra of nucleoli of the salivary gland (*Drosophila*) of the male (left) and of the female (right) (Schultz and Caspersson).

The maximum at 2600 Å is due to ribonucleic acid, while the second peak (about 2800 Å) is caused by *histones*, proteins rich in basic amino acids. We see that the nucleoli of the male are poorer in histones and that they contain relatively more ribonucleic acid than those of the female. Since heterochromatin is formed especially

in the heterochromosomes one can see how the latter would profoundly influence the chemical composition of the nucleolus. Schultz and Caspersson consider as do most geneticists (see Mather) that the heterochromatin is relatively inert from the genetic point of view, but that it may be the center of the synthesis of nucleic acids, thus controlling the reproduction of the genes.

Caspersson (1940) compared the chromatin of resting nuclei (salivary glands of *Chironomus*) with that of metaphase chromosomes (mitosis of maturation in the grasshopper). The euchromatin of the giant chromosomes is composed of thymonucleic acid associ-

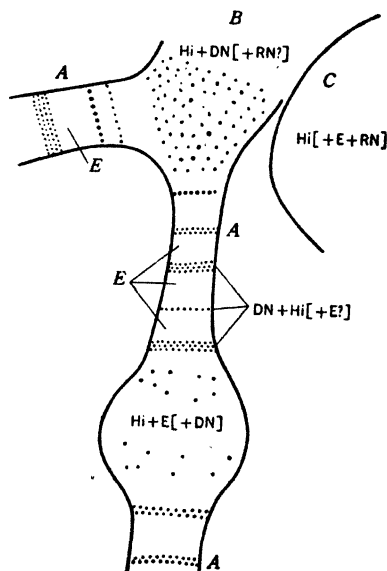


Fig. 51. Schematic structure of a chromosome (Caspersson): A, euchromatin; B, heterochromatin; C, nucleolus; Hi, histones; E, complex proteins; DN, thymonucleic acid; RN, ribonucleic acid.

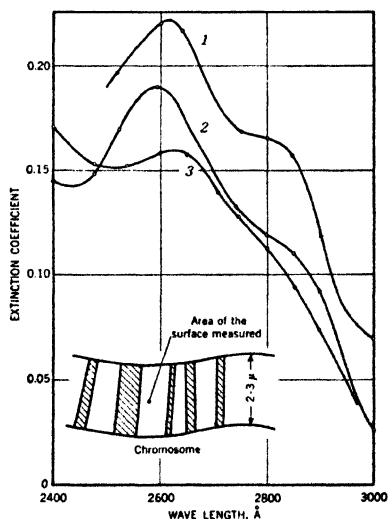


Fig. 52. Absorption spectra of various components of the giant chromosome (*Chironomus*): 1, 2, heterochromatin; 3, euchromatin. The band at 2650 Å is caused by nucleic acids (Caspersson).

ated with histones while the clear discs interposed between the chromomeres are lacking in nucleic acid and contain more complex proteins comparable to the globulins; heterochromatin has both types of nucleic acids\* and large amounts of histones and the nucleolus is

\* Caspersson considers the presence of ribonucleic acid in heterochromatin to be quite likely, and we have seen that Brachet's method has furnished the proof for it.

composed of histones associated with ribonucleic acid in varying proportions. The scheme presented by Caspersson in Figure 51 is a synthesis of these facts, while Figure 52 shows the absorption spectra of the various constituents of the resting nucleus.

This scheme can be extended to the molecular level by the observations of Mazia, Mazia and Jaeger, Frolowa, and Calvin, Kodani and Goldschmidt. These investigators studied the effects of nucleases, trypsin, and alkalies on the Feulgen reaction and on the ultraviolet absorption of giant chromosomes and they came to the conclusion that the chromosome is composed of a bundle of parallel polypeptide chains. This protein skeleton of the chromosomes is thought to be continuous, its integrity remaining when the nucleic acid is removed by various methods. The nucleic acid would be combined at certain points by the phosphoric acid groups to basic amino acids. This interpretation agrees with the observations of Caspersson who noticed an accumulation of histones rich in basic amino acids precisely at those places where thymonucleic acid is attached. On the contrary, it is contradictory to the hypothetical scheme of D. Wrinch who postulated a cyclical structure for thymonucleic acid, since we know now that its molecules are elongated in the native state.

Let us go on to the metaphase chromosome which shows an almost typical absorption spectrum of thymonucleic acid (Fig. 53). There is only a weak protein absorption probably due to the histones.

Mitosis is thus accompanied by a marked decrease in the amount of protein in the chromosome, and a proportional increase in its thymonucleic acid. While the ratio  $\frac{\text{thymonucleic acid}}{\text{protein}}$  is less than 1:10 in the resting nucleus, it attains a value of from 1:1 to 1:4 in the metaphase chromosome. A qualitative change also takes place such that during division the complex proteins disappear and only the histones persist. There would be, then, a simplification of the proteins of the chromosome during mitosis, comparable to that which has been observed by Kossel and Miescher in salmon spermatogenesis (where the muscle proteins disappear to give rise to the histone and protamine constituents of the sperm heads). It should be noted, however, that this conclusion of Caspersson does not appear to be rigidly demonstrated. The very intense absorption of nucleic acid in the metaphase chromosome masks the protein absorption; it would

certainly be very worthwhile to repeat the measurements on chromosomes which have lost their thymonucleic acid through the action of desoxyribonuclease. Also recall that Mirsky and Pollister, who have studied the absorption spectra of histones completely freed of nucleic acids, hold that it is impossible to distinguish spectroscopically

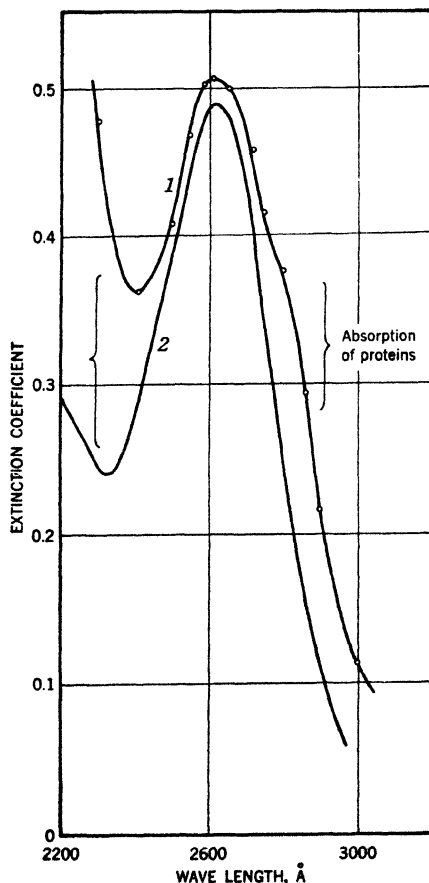


Fig. 53. Absorption spectrum of a chromosome in metaphase (1) compared to that of a solution of thymonucleic acid (2). The difference between the two spectra corresponds to absorption of the proteins (Caspersson).

between the basic histones and complex albumens. However, all the authors who have tried to determine arginine (an amino acid very abundant in histones) in sections agree that the reaction is particularly intense in the chromosomes (Serra and Queiroz-Lopes, Thomas, unpublished observations by Brachet). It seems to us that these observations support Caspersson's theory of a simplification of the

chromosome proteins during mitosis. It should be added that the cytoplasmic ribonucleoproteins (those which form the ergastoplasm, for example) also contain a high percentage of basic amino acids.

Caspersson arranges these facts in the following order: the resting nucleus contains thymonucleic acid, histones, and complex proteins; at prophase, thymonucleic acid accumulates in the chromomeres which carry the genes, and the complex proteins are hydrolyzed; at metaphase, the amount of protein in the chromosomes is considerably reduced; finally, at telophase, the synthesis of the products elaborated by the genes begins. These gene products are complex proteins in the euchromatin and histones in the heterochromatin. Simultaneously, the amount of thymonucleic acid in the chromosomes is lowered. The synthesis of histones by heterochromatin leads to the formation of the nucleolus which contains, in addition, ribonucleic acid. Part of these histones will diffuse out

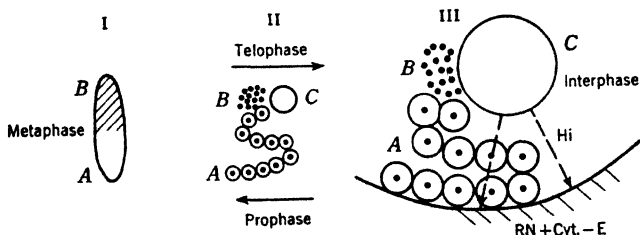


Fig. 54. Modifications undergone by nucleoproteins during mitosis; A, euchromatin; B, heterochromatin; C, nucleolus; Hi, histones; E, complex proteins; RN, ribonucleic acid (diagram from Caspersson).

of the nucleolus through the nuclear membrane and will stimulate the formation of cytoplasmic ribonucleoproteins which in turn will determine the synthesis of the proteins of the cytoplasm. This interesting concept is represented diagrammatically in Figure 54.

It will be seen that there is no incompatibility between this manner of picturing the facts and the writer's observations; the two types of observations complement each other to produce a unified theory of the changes undergone by the two nucleic acids and by the various proteins during mitosis.

One point remains completely obscure: why thymonucleic acid is strictly localized in the region of the chromosomes and is never found in the cytoplasm. Caspersson has tried to explain this point by



assuming that thymonucleic acid synthesizes the specific proteins of the genes as a result of its capacity to form elongated chains (a property not possessed by ribonucleic acid). He attempted to test this hypothesis by measuring the ultraviolet absorption of euchromatin, using polarized light, but he was unable to show differences, indicating that the molecules of thymonucleic acid are oriented parallel in the discs of the giant chromosomes. This conclusion has been recently confirmed (1944) by Frey-Wyssling. Caspersson remarks that there may be oriented chains too short to be detected by the methods used. This, of course, is hypothetical and the question still remains open.

Before leaving the problem of the localization of the nucleic acids in the cell, there remain some few questions relative to the chemical constitution of the chromosomes to be examined. The chromosomes are certainly composed for the most part of thymonucleohistone, but we may question whether this relatively simple substance can account for the extraordinary specificity of the genes. For this reason attention has been directed to the other components of the chromosomes, and there is no longer any doubt that isolated nuclei are not formed exclusively of thymonucleohistone. Thus Mayer and Gulick have isolated a protein containing much more sulfur than histone from the nucleus, while Stedman and Stedman have demonstrated the presence of an acid protein, which they call chromosomin, in chromatin. It is characterized by the presence of tryptophan which is almost absent in histones. Stedman and Stedman consider that chromosomin, which may form 40 to 50% of the dry weight of nuclei, corresponds to the genes. Unfortunately the British authors have expressed some manifestly radical views concerning the structure of the chromosomes, going so far as to deny the presence of thymonucleic acid. But they did draw attention to nuclear proteins other than histone and it seems well established now that chromatin contains one or more proteins rich in tryptophan. Jeener, in particular, showed that one can isolate from a concentrated NaCl solution of the nuclei some ultracentrifugable granules. These contain tryptophan and have a high phosphatase activity. Mirsky and Pollister have also demonstrated a protein rich in tryptophan in chromatin. The fact that isolated nuclei retain an appreciable respiration and the observations of Dounce showing that they contain several enzymes suggest that chromatin may con-

tain several distinct proteins possessing enzymic activity. It is well to remember that the high alkaline phosphatase activity of the nuclei has been demonstrated many times by the cytochemists (Gomori, Moog, Willmer, Danielli and Catcheside, Krugelis, *et al.*) and that this enzyme functions in the phosphate turnover in thymonucleic acid (Brachet and Jeener).

Nevertheless, these interesting observations should not cause us to minimize too much the possible role of thymonucleic acid in the structure and function of genes. Indeed one can not neglect the well established fact that when organisms are exposed to monochromatic radiations it is the wave length at which the nucleic acids absorb which is most effective in the production of mutations (Stadler, Hollaender). Therefore, it would seem that some change in the intimate structure of thymonucleic acid causes gene mutation and this fact makes it probable that the acid is an important component of the gene.

In addition, the recent observations of bacteriologists on the induction of changes in type in *Pneumococcus* (Avery and McCarty) and *E. coli* (Boivin and his collaborators) under the influence of polymerized thymonucleic acids after much purification, suggest the presence of a chemical specificity in the structure of these acids. The fact that depolymerization of these thymonucleic acids, by thymonucleodepolymerase, inactivates them completely indicates that the maintenance of the architecture of the native macromolecule is essential for obtaining the directed mutations in bacteria.

If these facts should acquire a general significance and if the existence of specific thymonucleic acids is really demonstrated one could explain why these acids are also the specific constituents of the chromosomes.

### 3. Role of Nucleic Acids in the Cell

The cytochemical findings just reported point irresistibly to the conclusion that nucleic acids must take part in the *synthesis of proteins*. Only this interpretation explains why the ribonucleic acid of the cytoplasm is always more abundant where protein synthesis is most intense; why the regions where mitoses are frequent are always rich in both types of nucleic acids; why yeasts, bacteria and viruses, which have great powers for multiplication, always contain large quantities of these acids and why the chromosomes always contain

nucleic acid. The observations of Caspersson and of Brachet are further supported by those of Kedrowski who has maintained that the basophilic proteins have a role in protein synthesis. This investigator did not know of their chemical nature and spoke of them only as "acid colloids," but he was struck by the role that they played in anabolism and gave them the suggestive name of *anabolites*. In his last work (1941) Kedrowski recognizes the identity of anabolites and the cytoplasmic ribonucleoproteins.

Caspersson has attempted to bring together into one coherent theory all the facts known about the function of nucleic acids during protein synthesis. His ideas may be briefly summarized as follows. There is a direct relation between the nucleic acids localized in the chromosomes, the nucleolus, and the cytoplasm. The most important center of the synthesis of proteins is the nucleus, particularly the chromatin. While the euchromatin regulates the synthesis of complex proteins in relation to the genes, the heterochromatin controls the formation of histones which accumulate in the nucleolus from which they diffuse into the cytoplasm. In the region of the membrane the histones stimulate the synthesis of pentosenucleoproteins, and the basic amino acids (arginine and histidine) give rise to the purines at this point. These cytoplasmic ribonucleoproteins in their turn stimulate the synthesis of cytoplasmic proteins. In short, the nucleoli and the nucleic acids of the cytoplasm constitute the intermediaries between the heterochromatin and the proteins of the cytoplasm (Fig. 55). It may be added that this hypothesis has been extended somewhat by Monné who considers that the euchromatin synthesizes the nuclear sap and regulates catabolism by its intervention while the heterochromatin insures the production of ribonucleic acid in the cell and thus controls anabolism.

The principal arguments advanced by Caspersson in favor of his theory are the following. There is a constant relationship between the presence of large nucleoli, rich in ribonucleic acid, and large amounts of cytoplasmic nucleoproteins. For example, the nucleoli are well developed in growing oöcytes, in nerve cells, glands, cancerous and embryonic cells while they are absent in cells which do not synthesize protein, such as cells undergoing spermatogenesis, segmenting eggs and leucocytes. The oöcyte and the neurone are good material for a more rigid analysis of the process. In the oöcyte, the Feulgen positive chromomeres are dispersed throughout the nu-

cleus, giving the appearance of a hypertrophy of the heterochromatin, while large nucleoli appear often numerous and, at the same time, containing a lot of histone. In the nerve cell, the nucleolus and the cytoplasm are very rich in ribonucleic acid, associated with histones.

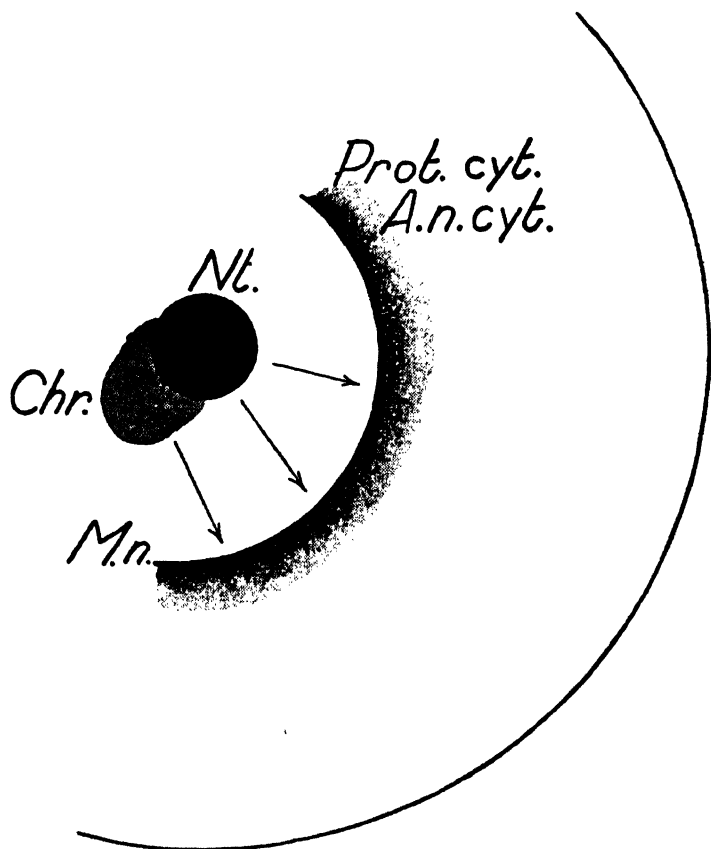


Fig. 55. Diagram of the passage of nucleic acid from the nucleolus (Nl.) toward the nuclear membrane (M.n.) where the cytoplasmic nucleic acids accumulate (A.n.cyt.). The latter synthesize the cytoplasmic proteins (Prot. cyt.) Chr., chromatin (Caspersson and Hydén).

According to Landström, Caspersson, and Wohlfart, and especially Hydén, the amount of histones in the nucleus diminishes from the nucleolus toward the nuclear membrane and the histones can be extruded from the nucleus through openings that occur locally in the

nuclear membrane. These observations were made on material fixed by the method of Altmann-Gersh (drying in vacuum at low temperature) in order to minimize the danger of artefacts. Whether artefacts were completely avoided still appears questionable, however. The neurone, like the oöcyte, shows a hypertrophy of the heterochromatin.

Caspersson, in collaboration with Brandt, has tried to verify his theory in the case of yeasts. Pressed yeast absorbs ultraviolet characteristically in the region of the "volutin" granules scattered in the cytoplasm. Starvation of the cells by agitation in distilled water significantly reduces the number of these granules. If, on the contrary, yeast is placed in a medium containing a nitrogen source, assuring rapid growth, the volutin granules dissolve in the hyaloplasm and its content of nucleic acid increases considerably. Thus the cell during growth is more homogenous and absorbs ultraviolet more intensely than when in a resting condition. When yeast is placed in a medium favorable for intense respiration or fermentation, the character of the volutin granules does not change. Therefore the synthesis of nucleic acid occurs only with the growth of the culture and is not influenced by the rate of carbohydrate metabolism. Caspersson expresses the opinion that the nucleus of yeast, which is very small and contains thymonucleic acid, is homologous with euchromatin while the volutin granules correspond to the heterochromatin which in the lower organisms is localized in the cytoplasm. The dissolving of these granules during growth would be the equivalent of the dissolution of the heterochromatin in the neurone and in the oöcyte at the time when they synthesize proteins. Bacteria no doubt behave in the same manner as yeasts in this regard.

This interesting theory deserves critical discussion. First of all it will be remembered that the parallel between the basophilia of the cytoplasm and that of the nucleolus shows some exceptions notably during oögenesis and embryonic development. However, these exceptions are few in number and it is probably possible to explain them if Caspersson's theory is slightly modified.

A few remarks concerning the technique, the choice of material, and the interpretation of certain results, is appropriate at this point. Caspersson's method is still, in spite of its refinements, a cytochemical technique. It gives remarkably precise information concerning the *localization* of the compounds being studied and some indica-

tion of their quantities, but because of its necessarily static character not much is learned about the nature of the metabolic changes which the nucleoproteins undergo. As a result of this, Caspersson's interpretation of the facts becomes hypothetical just at the point when he tries to translate them from a descriptive and topographical level to a physiological one. For example, Caspersson concludes that a simplification of the protein constituent of the chromatin occurs during mitosis and he compares his observations with those of Kossel, Miescher, etc. on spermatogenesis of the salmon. These authors showed by complete chemical analysis that the muscle proteins undergo autolysis and that the arginine derived from this hydrolysis takes part in the formation of the protamines of the spermatozooids. Caspersson naturally did not use the same methods and he compares the chromosome of the spermatocyte with the nucleus of the salivary gland. It may well be asked if this comparison is valid, drawn, as it was, from a comparison of cells of different organs and species. The question of whether the nucleus of the salivary gland, with its very particular structure, is a typical resting nucleus is also disputable. Finally, it should be remembered that the ultraviolet absorption of chromosomes in metaphase is so intense that Caspersson can not be very positive on the nature of the protein combined with thymonucleic acid. The demonstration would certainly be convincing if the comparison had been carried out on resting cells and on chromosomes of the same organ and, if possible, if it had been done on the same microscopical preparation. Thus, Caspersson's idea of a hydrolysis of complex proteins in the metaphase chromosome has not yet been firmly established experimentally.

A still more serious objection already noted above has been offered against Caspersson's theory by Mirsky and Pollister and by Mirsky (1943). According to these investigators, the absorption spectra of purified histones extracted from nuclei of different origins can not be distinguished from those of more complex proteins (albumins, for example). If this is true, a distinction between histones and complex proteins is impossible by Caspersson's method and serious doubt is thus cast upon Caspersson's statement that the different segments of the giant chromosomes are made up of distinct proteins and that a simplification of the proteins occurs during mitosis. However, we have seen that the observations by Serra and Queiroz-Lopez and those of Thomas on the localization of arginine in the

resting cell and in mitosis indicate that, in agreement with Caspersson's theory, the chromosomes must be rich in basic proteins of the histone type.

The recent results obtained by Brunberg and Larionow tend, at first sight, to instill some doubt on the validity of Caspersson's method concerning the possibility of detecting the nucleic acids. Using a new type quartz objective the Russian authors were able to take photographs in the ultraviolet of cells cultivated *in vitro* without killing them. Under these conditions, they found no selective absorption at 2600 Å by either the nucleus or the nucleolus. But if the cells are killed by prolonged exposure to ultraviolet or by chemical agents, Caspersson's results are then obtained. These interesting results do not mean that there are no nucleic acids in the nucleus of the living cell and it is not necessary to consider them as opposed to Caspersson's results. However, they indicate, if correct, that thymonucleic acid in the living cell is present in a different form than previously recognized. Undoubtedly, the purine and pyrimidine bases may be combined in such a manner that they do not absorb U.V. We await with interest the confirmation of these observations and point out that the phenomenon observed by Brunberg and Larionov is not without parallel; thus Crammer and Neuberger have reported that native egg albumen contains hardly any phenol groups of tyrosine in ionized form and thus this protein does not absorb U.V. below 2850 Å. However, denaturation with alkalis, heat, urea, etc. causes the characteristic absorption spectra of tyrosine to appear; the denaturation probably brings about the rupture of hydrogen bonds. Also it must be remembered that some time ago Vlès and Gex found that the living sea urchin egg did not give the characteristic protein absorption spectra, but that this absorption appeared during cytolysis. Beyond doubt facts of this nature have a great interest and they deserve a careful study.

If it is true that thymonucleic acid does not absorb in the U.V. in the living cell, a certain caution must be exercised in the use of Caspersson's method as a quantitative measurement. The nature of the fixing agent used may be a factor which must be taken into account. Along the same line of thought, it must be pointed out that there are indications of the existence of enzymes able to deaminate the purines in the intact nucleic acid (Greenstein and Chalkley; Örström); since the absorption spectra of the hydroxypurines differ

measurably from that of aminopurines, the deamination of a nucleic acid is followed—using Caspersson's method—by a decrease in the absorption which one would naturally interpret as a drop in the amount of nucleic acid. In the case of the sea urchin, for example, Öström has shown that the deamination of the purines of ribonucleic acid is considerable at fertilization. It is probable that the U.V. absorption at 2600 Å also decreases at this time without any real decrease in the amount of nucleic acid. Thus we see that a number of factors must still be examined before the method of microspectrophotometry can be used quantitatively with complete assurance.

It will be recalled that one of the great dangers the cytochemist must avoid is defective fixation of the cells and tissues that he is investigating. This danger does not appear to be important in this particular case, for proteins are easily fixed by usual methods; the technique of Gersh, adopted by Caspersson, is, in principle, a favorable one. One would like to know, nevertheless, how the nucleases resist these manipulations. Now, Gersh's method consists in desiccating the tissue at low temperatures and infiltrating it directly with melted paraffin; ribonuclease probably resists this infiltration, since it survives boiling, and if it is not inactivated by freezing and drying one runs the risk of having the enzyme functional, bringing about the pictures of diffusion of the nucleolar nucleoproteins described by Hydén in the nerve cell, for example. Incidentally, it is impossible to say exactly how such a migration occurs, if it does occur. Hydén believes, as Caspersson does, that nucleoproteins come out of the nucleolus toward the nuclear membrane, but the reverse idea, that the cytoplasmic nucleoproteins diffuse through the nuclear membrane and converge in the nucleolus, can not be completely excluded. It has so often been erroneously concluded from simple microscopic examination that a migration of a substance or of cells takes place, that caution must be exercised.

There is a matter of detail in Caspersson's theory that appears particularly dubious. This is the homology established between the volutin of yeasts and the heterochromatin of higher forms. In fact, we have seen above that the tendency for cytoplasmic ribonucleoproteins to become disseminated into fine granules during mitosis is very general. But Caspersson bases his identification of the volutin of dividing yeast cells with heterochromatin chiefly on its resolution and, on this basis, there is no reason for not placing in this category



the cytoplasmic ribonucleoproteins of higher organisms. Furthermore, heterochromatin always contains thymonucleic acid which is missing in the volutin and it appears more reasonable that the heterochromatin in yeast as in other forms would be localized in the nucleus.

The chemical evidence at our disposal does not permit judgment of Caspersson's theory, for all that can be said is that the germinal vesicle contains only a small quantity of peptidases which one would logically suppose take part in protein synthesis (see Chapter III). However, according to Caspersson, this nucleus shows all the signs of hyperactivity of the chromatin making it an especially active center of protein synthesis. Duspiva was able to show a relation between the dipeptidase activity of the cytoplasm and the formation of yolk, but he saw no correlation between this latter process and the peptidase activity of the nucleus. We must conclude that if the germinal vesicle is actually the center of protein synthesis in the oöcyte, it exercises its control by means of chemical reactions of which we are as yet ignorant. It is at this point where Caspersson's theory presents the greatest difficulty. The relation between nucleic acid and protein synthesis appears to be clear on a morphological basis but we do not understand the chemical relations of such a tieup.

In connection with yeast the writer has, in collaboration with R. Jeener, made some observations which do not agree entirely with those of Caspersson and Brandt. If the growth of yeasts which have been "starved" by treatment with distilled water and placed in a mineral medium is studied, a synthesis of nucleic acid is seen. This synthesis like growth itself is linear (Fig. 56). But we see that the increase in amount of nucleic acid in the yeast is much more rapid than its multiplication and the latter, furthermore, is not increased by the increased nucleic acid in the cells. Nevertheless, we would expect such an increase if there were a direct relation between growth and nucleic acid.

On the contrary, we find that the amount of nucleic acid can vary widely before there is any detectable effect on growth. Boivin and Vendrely came to the same conclusions in the case of bacteria. The determinations of the purines by the French authors indicate that the bacteria show an increase in nucleic acids during the time before division, that is to say when each bacterium is undergoing a marked growth; during the division which follows the increase in size

of the bacterium, the nucleic acid content tends to decrease. Thus bacteria behave in exactly the same fashion as cells where the synthesis of proteins goes on parallel with the synthesis of ribonucleic acid and where this acid tends to disappear during mitosis.

These remarks are not made to impugn Caspersson's theory but only in a cautionary way. Caspersson's concept is very ingenious and takes into account a number of facts known to cytologists which have remained unexplained. It is based on an imposing array of cytochemical findings which should be extended. The deductions which Caspersson has drawn from them are often daring, but they seem to constitute a fair approximation of the real state of affairs and form a useful basis for future research.

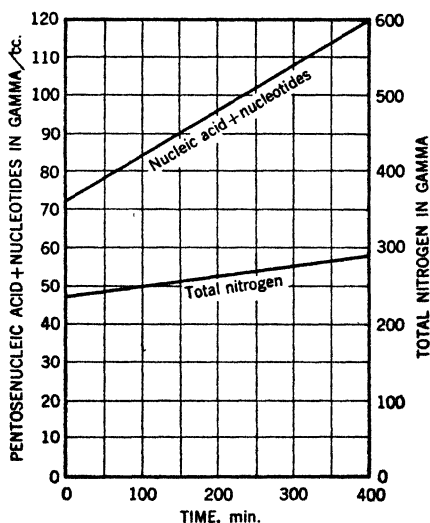


Fig. 56. Increase in basophilia and total nitrogen (growth) in "starved" yeasts placed in a mineral medium (Jeener and J. Brachet).

The principal difficulty encountered in considering these ideas consists, as we have seen, in the impossibility of furnishing biochemical proof for the relation existing between nucleic acids and protein synthesis. It was with the purpose of elucidating this point further that the writer attacked the problem from another angle, in collaboration with R. Jeener. These experiments stem from some interesting work by A. Claude devoted to the study of the virus of transmissible tumors in birds. By ultracentrifugation of homogenized tumor, Claude isolated small particles visible under the ultra-microscope which were highly infectious and which consisted of a

ribonucleoprotein associated with lipids. He quickly noticed that particles almost identical save for virulence were obtained from chick embryos and normal mammalian tissue. Stern later established that these granules were also present in cardiac muscle and that they contained important respiratory enzymes, notably cytochrome oxidase and succinic dehydrogenase. According to Lazarow, these particles are also capable of oxidizing glutamic acid rapidly. With the aid of the electron microscope, Stern (1943) was able to establish that they have a spherical shape while Claude was able to observe some details in their structure.

Jeener and the writer have shown that it is possible to isolate analogous granules from widely different organs of both vertebrates and invertebrates and also from yeasts and plants. Their abundance is, by and large, proportional to the amount of ribonucleic acid in the organ examined and their composition is rather constant. Ribonucleic acid, sulfur proteins, lipids of the "plasmalogen" type and enzymes (indophenol oxidase, peroxidase) are regularly found. Quantitative differences are nevertheless found between one type of material and another. It is important to emphasize that almost all of the ribonucleic acid present in the extracts is bound to the granules, the supernatant liquid after ultracentrifugation containing only traces of this substance. This observation has also been made by Claude (1944, 1946). This rule, however, applies only to adult organisms; in the eggs of amphibians, on the contrary, the granules contain only 20 to 40% of the ribonucleic acid present in the extract (J. Brachet and H. Chantrenne). The remainder is found in a "free" form, which is nonultracentrifugable. The same holds true for the chick embryo and, as we shall see presently, for yeast.

These granules contain practically no nuclear debris and, in fact, are free of thymonucleic acid even when isolated from an organ rich in nuclei, such as the thymus. Furthermore, only a very small number of particles are obtained when hemolyzed red blood cells of birds are homogenized and centrifuged and these are composed almost exclusively of nuclei (personal communication of R. Jeener). Finally, the granules are also free of glycogen.

One can demonstrate without difficulty the pre-existence of these granules in the living cell. If an entire organ—for example, the frog's liver—is ultracentrifuged (Brachet and Jeener, Claude) then it is seen that ribonucleic acid which was distributed throughout the

cell is concentrated at the centrifugal pole of the cell. It is accompanied during this migration by the sulfhydryl proteins, the plasmalogen, the peroxidases, and indophenol oxidase; all this can be easily demonstrated cytochemically. It must be assumed that all the constituents of the granule are thus associated into a complex in the living cell, since their molecular weights differ considerably. Furthermore, the particles are visible under the ultramicroscope in certain very favorable situations, such as the cells of the silk glands of caterpillars. They ordinarily escape detection in the living cell probably because their index of refraction is so close to that of the hyaloplasm. The identity of these granules with mitochondria, at first suggested by Claude, appears now to be excluded.

The further investigations of Claude (1943) have demonstrated that these granules, which he calls microsomes, are in reality distinct from mitochondria, for the latter are much larger and have less lipids and less phosphorus than the microsomes (Claude, Lazarow). The latter are richer in ribonucleic acid than the mitochondria or the secretory granules (which have been isolated from pancreas and liver and analyzed by Claude); they are responsible for the basophilia of the hyaloplasm. The fact that the secretory granules break up in distilled water into granules similar to microsomes leads Claude to believe that secretory granules originate from the microsomes. Nevertheless, in certain cases there are quantitative chemical differences between these two types of particles: for example, *d*-amino acid oxidase is present in the secretory granules of the liver but is absent in the microsomes; succinic dehydrogenase, according to Hogeboom, is essentially a constituent of the mitochondria and it is without doubt absent from the microsomes. Recently, Moog and Steinbach, as well as Chantrenne, have shown that in reality one can isolate by fractional centrifugation a large number of granules having different sedimentation constants. It is the larger granules that contain the most phosphatase in both the chick embryo (Moog and Steinbach) and in mouse liver (Chantrenne). In addition, Chantrenne has found that these large granules contain less ribonucleic acid than the small ones.

A particularly interesting case, studied by Steinbach and Moog, is that of the enzymes hydrolyzing adenosine triphosphate (ATP). There are two distinct enzymes known, an apyrase which removes two phosphates from the substrate and an ATP-ase (adenosine tri-

phosphatase) which only liberates the terminal phosphate. Steinbach and Moog found that the two enzymes were present exclusively in the granules. That is to say that the supernatant liquid did not contain them after ultracentrifugation. However, it was possible to separate the apyrase and the ATP-ase by a fractional ultracentrifugation since the two enzymes are bound to particles of different size. The presence of these enzymes in the granules along with cytochrome oxidase and succinic dehydrogenase is a very remarkable coincidence. Indeed the hydrolysis of ATP liberates large amounts of energy (like oxidations) and it seems more and more certain that this reaction furnishes the energy necessary for a number of biological syntheses.

We have tried to determine the presence of hydrolytic enzymes in the isolated granules and a whole series of hydrolytic enzymes has been identified easily: phosphatase, amylase, trypsin, cathepsin, dipeptidase, ribonuclease, adenylic deaminase. However, a large proportion of these enzymes is also found in the supernatant liquid and is not bound to the granules as specifically as is ribonucleic acid. The enzymes are bound in various degrees in any particular case. In addition, the granules from the hypophysis contain some of the hormone for the expansion of the melanophores; similarly, insulin and haemoglobin are found in the particles isolated from pancreas and red blood cells, respectively.

The respiratory enzymes in yeast have been studied by H. Chantrenne and he has confirmed the presence of cytochrome oxidase and succinic dehydrogenase which are specifically fixed to the granules. Other dehydrogenases, on the contrary, are divided between the granules and the supernatant liquid. Chantrenne tried to determine the exact physiological role of these granules by ultracentrifuging the intact liver of the frog. A marked decrease in the oxygen consumption was observed and this decrease was almost entirely in the fraction which is cyanide-sensitive. Thus, sedimentation of the granules has the same effect on respiration as cyanide. This is easily understood when it is noted that ultracentrifugation throws the granules with their cytochrome oxidase to the centrifugal pole of the cell and mechanically isolates them from the rest of the constituents of the chain of oxidations. The experiment thus permits the spatial separation of the respiratory enzyme from its substrate, a separation which Runnström has held to be responsible for the low respiration of the

unfertilized sea urchin egg (see Chapter IV). The experiments of Chantrenne explain why the cyanide-sensitive respiration is specifically lowered by ultracentrifugation in the eggs of *Ascaris* (Huff and Boell).

In resume, there are in the cell particles possessing a certain unity of chemical composition and specifically carrying ribonucleic acid, cytochrome oxidase, succinic dehydrogenase, ATP-ase and apyrase. Various hydrolytic enzymes and desmolases are also associated with them, certain of them (alkaline phosphatase, dipeptidase) being firmly bound to the granules.

In attempting to clear up their role in the cell, the experiments that Brachet has carried out with Jeener on yeasts are helpful, since these cells contain, contrary to adult organs, a high proportion of "free" ribonucleic acid which is not a part of the granules. We know that it is possible to lower the amount of nucleic acids in yeasts considerably when they are cultured in a salt medium free of phosphorus. The addition of phosphate stimulates a sudden increase in the basophilia which increases 5- to 10-fold in an hour. These facts have already been noted by van Herwerden, and we have seen that they contradict the hypothesis of a simple relation between the nucleic acid content and growth. A closer examination demonstrates that these large variations in the amount of ribonucleic acid of yeast are obtained only in the "free" fraction and that the composition of the granules on the other hand remains remarkably constant even when the concentration of the free ribonucleic acid of the cell increases tenfold in less than an hour. These considerable deviations hardly modify the number of granules in the yeast cell at all. To all appearances, the number of granules increases in proportion to the growth of the culture. From this it is clear that there would be no direct relation between the multiplication of yeast cells and their ribonucleic acid content, since this relation is only true for the granules. On the contrary, free nucleic acid appears to be a much more labile fraction which may have a very different physiological role. Jeener and the writer have found that the synthesis of free ribonucleic acid in the presence of phosphate does not take place unless the carbohydrate metabolism of the cell is intact. This fact leads us to think that this fraction can play a role as an "energy reserve" similar to adenosine triphosphate and phosphagens.

The analysis of these phenomena has been pursued in our lab-

oratory by J. Wiame, who showed that the recharging of the phosphate in yeast cultivated in a mineral medium free of this element is accompanied by a phosphorylation of the zymonucleic acid rather than a true synthesis of this acid. Under these conditions a compound containing 15% phosphorus is formed, although zymonucleic acid contains only 9.5%. The properties of this substance indicate that it must contain a polyphosphorylated nucleic acid of the adenosine triphosphate type. The later investigations of Wiame indicate that it must be a polymer of a hexametaphosphate, and the ultimate relation to zymonucleic acid remains to be determined. The synthesis of this compound is coupled with the catabolism of the carbohydrates, probably with the oxidation of triosephosphates. Now, this is a characteristic common to numerous biological phosphorylations (Lynen, Kalckar, Mann).

The center of interest concerning the synthesis of proteins thus shifts from nucleic acid to the granules. Ordinarily, the free fraction is quantitatively less important and it is obvious that the cytochemical methods used by Caspersson and by Brachet demonstrate the localization of these particles. Now, there is no proof that nucleic acid as such takes part directly in the synthesis of proteins; hence, it may well be that the latter are elaborated by the other constituents of the granule; for example, by the joint action of the proteases and the respiratory enzymes.

Without attempting to discuss in detail here the possible mechanisms in the synthesis of proteins which have been considered at length (Brachet 1946), let us point out certain essential facts. The work of Bergmann and his collaborators has established that proteases can catalyze the synthesis of a peptide bond. This synthesis is not possible *in vitro*, however, for thermodynamic reasons, unless the products formed are insoluble and removed from the sphere of action. One must therefore suppose, with Linderstrøm-Lang and Fruton, that the cell which synthesizes proteins eliminates the product of the reaction continually by rendering it insoluble. It is also possible that the cell, by furnishing energy through oxidations or by the hydrolysis of the energy rich bonds in ATP, compensates for the endothermic character of the reactions involving enzymic synthesis. The existence of a coupling between the oxidations and the synthesis of the peptide linkages is shown by the recent investigations

of Cohen and McGilvery on the synthesis of *p*-aminohippuric acid by tissue slices. This peptide does not form unless the cells are actively respiring.

We see immediately that the granule appears as an ideal organelle for the synthesis of proteins: these particles correspond to the mitochondria or ergastoplasm of the cytologists, which we know play a role in the secretion of yolk or of the secretory granules; they contain some cathepsin and some peptidases which can synthesize the peptide bond when circumstances are favorable; these enzymes are intimately associated with respiratory enzymes and the apyrases which insure the production of energy indispensable to an endothermal synthesis. Finally, the granules contain, in small amounts, the proteins which they synthesize. Thus, we explain the facts that the granules of red blood cells contain a small amount of the haemoglobin present in the cell and that this haemoglobin is very firmly bound.

How shall we interpret the constant occurrence of ribonucleic acid in the granules? It could be imagined that this substance forms a simple "cement," uniting the various enzyme molecules which form the granule, but this hypothesis becomes improbable by reason of the fact that the granules do not dissolve when their nucleic acid is broken down with ribonuclease. This treatment does not modify the number, the shape, or the appearance of the granule and thus we must conclude that the ribonucleic acid is found at the periphery of the particle.

The fact that the nucleic acids have been found in all living organisms, including obligatory parasites such as the viruses, makes it very probable that these acids play a role in synthetic processes; this supposition is supported by the fact that the wave length 2650 Å, at which the nucleic acids absorb strongly, is most effective in preventing growth in virus (Price and Gowen), in yeast (Oster), in mushroom spores (Hollaender and Emmons), and in bacteria (Giese). We have seen in the preceding chapter that the nucleic acids or their derivatives often act as growth factors and that substances which inhibit the synthesis of nucleic acids also stop growth in microorganisms. All these facts remain inexplicable unless we assign to the nucleic acids a role in processes involving synthesis. It must be confessed, however, that the manner in which this functions still remains obscure and that one is reduced to speculation on this subject.



One of the most interesting theories about this question, proposed by Friedrich-Freksa, concerns more particularly the case of the gene. Assuming that the basic amino acids such as arginine and histidine have a precise location in the protein molecule, nucleic acid will then combine with them, the acid groups reacting with the—NH<sub>2</sub> groups of the amino acids. Thus a template of the specific protein is formed and this nucleic acid with its specific structure will later react with amino acids which are forced in turn into the same positions as in the initial protein. It must be repeated that this is only a hypothesis supported solely by the fact that arginine is the only amino acid present in all proteins.

Another explanation resembling the above has been suggested by Rondoni. This author assumes that the location of the electrical charges in the nucleoprotein determines where the amino acids which build up the new protein molecule will arrange themselves, and that the proteases will then unite these amino acids to each other.

Finally, Chantrenne (personal communication) has made the observation that nucleic acids precipitate numerous proteins; Warburg and Christian have actually taken advantage of this property to purify certain enzymes. Thus we can assume that nucleic acid serves to precipitate the products synthesized by proteases. The equilibrium of the enzymic reaction will thus be constantly displaced in favor of synthesis. This explanation has the advantage of being more amenable to experimental verification than the preceding hypotheses.

A particularly attractive hypothesis which is supported by certain experimental data was presented by Spiegelman. If yeast cells are forced to ferment a foreign sugar such as melibiose or galactose, a synthesis of an "adaptive enzyme" of a protein nature (melibiozymase or galactozymase) takes place. By studying phosphorus metabolism (using radioactive P) in such yeast, Spiegelman and Kamen observed that there is a deficit in labelled phosphorus in the nucleoprotein fraction. This transfer of nucleoprotein phosphorus does not occur when the yeast ferments glucose and thus is not synthesizing the adaptive enzyme. Any agent that inhibits the synthesis of proteins or adaptive enzymes also inhibits the transfer of P from nucleoproteins. These facts led Spiegelman to the following hypothesis on the role of the nucleic acids in the synthesis of proteins. The phosphate compounds able to store energy, ATP for

example, transfer this energy to specific "energy donors." In the case of synthesis of proteins and enzymes the nucleic acids may be the specific energy donors.

It is probable that the participation of nucleic acids in the reproduction of genes and viruses as well as in the synthesis of proteins will not become clear until such time as we have at our disposal exact determinations of the structure of these molecules. The studies of Butenandt, Friedrich-Freksa, Hartwig and Scheibe on the crystalline structure of tobacco mosaic virus have established the fact that ribonucleic acid is attached in a definite manner to the proteins. The purines and pyrimidines are oriented parallel to each other and are arranged like a stack of coins perpendicular to the long axis of the molecule.

We must not exclude the possibility that histones combined with nucleic acids take part in the synthesis of proteins. We are reminded that Edlbacher, like Friedrich-Freksa, considered that arginine plays a major role in the synthesis of the proteins of the gene. It is, however, doubtful whether arginase participates as actively as Edlbacher thinks. In fact, this enzyme is absent from the nuclei of frog oöcytes, and is lacking as well in cytoplasmic granules of tissues, with the exception of those derived from the liver.

Thus, the question of the relation between nucleic acids and the synthesis of proteins is far from being solved, but the problem is now clear and some important steps in its solution have been taken. The observations made on granules, especially, take some of the mystery from Caspersson's theory; nucleic acid no longer appears as the mysterious *deus ex machina* but as one of the functional components of an organelle designed for synthesis of proteins. The gap separating the morphological, cytochemical, and biochemical evidence is thus partially closed.

It may be repeated here that, undoubtedly, the role of nucleic acids in the cell is not limited to the one just discussed. We have already seen that they probably also constitute an energy reserve which can be utilized in phosphorylating reactions. The observations of Ostern and of some of his collaborators suggest further that ribonucleic acid in yeast gives rise to the mononucleotides taking part in glycolysis. This origin has been demonstrated for muscle adenylic acid and adenosine triphosphate and it is probable that these nucleotides contribute to the formation of the coenzymes for dehydro-

genases. Let us remember, finally, that, according to Örström, the nucleic acids of the sea urchin egg are deaminated during fertilization and that the ammonia formed increases the metabolism; it is not impossible that it also contributes to the synthesis of amino acids. We shall return once more to the nucleic acids in connection with other important phenomena which are intimately concerned with morphogenesis.

#### 4. Conclusions

The presence of ribonucleic acid in animal cells is definitely established, and its localization in the cell is no longer a matter of doubt; we find it associated with histones in both the nucleolus and the cytoplasm, and chromatin, especially heterochromatin, contains a certain proportion amount of it. Ribonucleic acid is particularly abundant in the ergastoplasm of gland cells and young oöcytes. In general, it is found wherever vigorous protein synthesis is carried on, while organs like kidney and muscle contain very little. It is probable that in these cases, too, they play a role in the synthesis of proteins, but here it is a matter of the formation of the proteins of the organ itself which we know are undergoing a continual replacement. The differences between the pancreas and muscle, for example, are thus simply quantitative; the amount of pentose, in these two organs varies in proportion from 8 or 10 in the former to 1 in the latter.

The manner in which nucleic acids take part in protein synthesis is not yet clear. The investigations of Caspersson and Schultz show that the content of thymonucleic acid in the nucleus regulates the amount of ribonucleoprotein in the cytoplasm. The observations of Caspersson and of Hydén suggest the presence of a complex mechanism operating in such a way that the euchromatin synthesizes the specific proteins through which the genes exercise their action while the heterochromatin elaborates the ribonucleoproteins of the nucleolus which then induce the synthesis of cytoplasmic nucleoproteins. These latter, finally, are the factors in the synthesis of ordinary proteins.

The ribonucleoproteins of the ergastoplasm are bound to granules or they coexist with some proteases and some oxidative enzymes; these various substances probably collaborate to synthesize proteins: the amino acids might be arranged on the surface of the granule in a precise pattern, attracted by the electrical charges of these various

compounds. The proteases then unite them, with the aid of oxidative enzymes and also of nucleic acid, which orients the reaction in the direction of synthesis. This explanation is hypothetical at present, but we may hope that the question will become amenable to experiment in the near future.

Cell division profoundly affects the nucleoproteins. It is accompanied by a synthesis of thymonucleic acid and probably by a destruction and a simplification of the proteins. Simultaneously, the nucleolus disappears and the ergastoplasm becomes resolved into fine granulations dispersed in the cellular space. The achromatic figure often takes up ribonucleic acid while the amount of this acid in the cytoplasm decreases. The chromosomes become richer in ribonucleic acid which perhaps contributes to the synthesis of thymonucleic acid.

Let us return now to the developing egg; we should expect to see the ribonucleic acids undergo a complex change, with a part of them being used in the synthesis of thymonucleic acid. However, if at the same time there is also an elaboration of proteins, an increase in the ribonucleoprotein content should occur. One can predict that this synthesis of cytoplasmic nucleoproteins will take place especially at the time when cellular differentiation occurs, for it is then that the proteins characteristic of each organ are built up. It will also be striking in certain cells having a secretory function, this being the case in the digestive tract when it becomes functional.

We will return later on to the various aspects of the question in relation to amphibian eggs and, for the moment, limit ourselves to a discussion of the prototypes of the two methods of synthesis of thymonucleic acid: the developing sea urchin egg and the chick embryo. As we have seen (Fig. 41), the cytoplasmic basophilia decreases little by little during the course of development of the sea urchin egg and this fact implies a drop in the amount of ribonucleoproteins corresponding to their utilization for the synthesis of thymonucleic acid. Nevertheless, if we examine the plutei we observe that their digestive tract is conspicuous by its very marked basophilia, although the ribonucleoproteins continue to decrease in the ectoderm and the mesoderm. But this is the stage when the larva begins to feed and it is logical to assume that the cells of the digestive tract secrete digestive enzymes at this time, either intra- or extra-cellular. The two pro-

cesses of synthesis and of utilization of ribonucleic acid thus coexist in the pluteus although only its destruction was detected in preceding stages.

Thus we see that the transformation of ribonucleic acid into thymonucleic acid would be detectable only in eggs where the formation of proteins is relatively reduced and these conditions are naturally not realized in the egg of the hen where the embryo continues to grow at the expense of the yolk mass. This embryo is naturally rich in ribonucleic acid, since it is the site of a continual protein synthesis. Cytochemical examination confirms this and Figure 57 shows a section of a 20-hour chick embryo stained to show the nucleic acids. The cytoplasm is deeply stained, but we note that the amount of ribonucleoproteins in the dividing cells is greatly decreased. In these cells the utilization of ribonucleic acid exceeds its synthesis. In

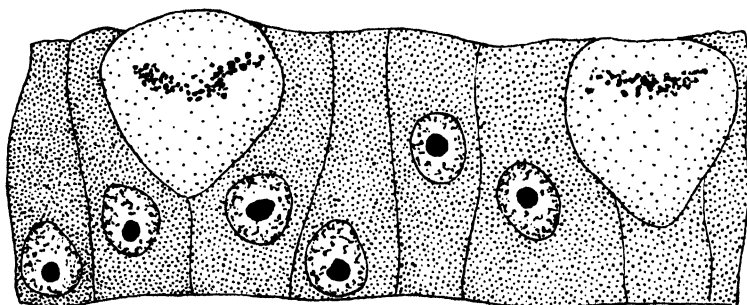


Fig. 57. Young chick embryo: cytoplasm of the cells in mitosis has a lower ribonucleic acid content (J. Brachet).

summary, we have come to think of eggs with partial synthesis and those with total synthesis as differing only quantitatively. In the first, catabolism of the ribonucleic acid exceeds its anabolism, while the contrary is true of the second because they are the site of an intense protein synthesis.

One point remains to be discussed. This is the respective roles of the ribonucleic acid in the "free" fraction, and that which is found to combine with the granules. These latter evidently take part in protein synthesis. We also know that "free" ribonucleic acid is found in appreciable amounts only in those cells which are in a state of active proliferation such as those of yeast, and chick and frog embryos. In the case of yeast, at least, there is no sign that the free ribonucleopro-

tiens take part in the synthesis of proteins. The conclusion is reached that this "free" fraction is very labile and participates in the synthesis of thymonucleic acid and in the production of the coenzymes for phosphorylation. This assumption gains credence from the fact that the amount of free ribonucleoprotein drops progressively during development, just as does the frequency of mitosis. The study of favorable material, such as the egg of the sea urchin, would permit one to see if such a hypothesis has a foundation in fact.



## CHAPTER VII

# Growth, Differentiation, and Metabolism

One may conceive of embryonic development as the result of three more or less independent processes, each of which requires energy for its realization: simple *maintenance*, which permits the egg to maintain its structure intact; *growth*, which implies a synthesis of protoplasm; and *differentiation*, which modifies the form of the embryo. It is possible to a certain degree to separate these three properties of the developing egg and thus to investigate where the energy comes from for embryonic growth and differentiation. The very controversial question of the energy of growth and of differentiation has been discussed at length by Rapkine (1928) and especially by J. Needham (1931, 1933); as the problem has made little progress since that time we will refrain from going over the arguments except to discuss the original contributions that Tyler has made to the subject.

The *maintenance* energy serves only to maintain the organism in equilibrium with the external environment; it corresponds to the basal metabolism of the adult individual at rest; in embryology the mature, unfertilized egg is in equilibrium with the external environment and shows neither growth nor differentiation, the energy it uses going entirely into its maintenance. It sometimes occurs in development that growth and organogenesis stop for a more or less prolonged period. This happens during the diapause in certain insects; for example, the mitotic activity stops completely and the oxidations of the egg are no longer used, except for maintenance. Development can also be blocked experimentally by cold, anaerobiosis, acidity, starvation, addition of dinitrophenol, etc; when the blockage is reversible, a situation very similar to diapause is obtained.

It is often possible to stop growth without causing the death of the cell; one obtains this result with bacteria treated with  $\text{HgCl}_2$  or



with sulfanilamides. In the first treatment it suffices to add cysteine, and in the second *p*-aminobenzoic acid in order to restore growth that has fallen to zero (Fildes). But it does not always work out this way. When one provides an insufficient diet for young animals they stop gaining weight and die very soon if growth remains inhibited. However, if normal nutrition is given in time, they very rapidly overtake the controls, making up their "weight debt" (Thompson and Mendel). Organisms capable of surviving very low temperatures or a pronounced dehydration are in a state of very low metabolism, corresponding to their maintenance energy.

It is difficult to define *growth*. It consists in transformations of foods into protoplasm. Thus we may say that growth occurs when the embryo uses its yolk, even when the size of the entire egg does not change. We may have growth without differentiation, such as takes place in *anidic* embryos (Dareste, Grodzinski) where development consist solely in an increase in size; morphogenesis is lacking because of a defect in the organizer. The same result is obtained experimentally by irradiating the organizer center with ultraviolet light (Dürken) or by explanting a fragment of the ectoderm in the amphibian (Holtfreter).

Under the heading *differentiation* we include all the morphological changes which characterize development and also the elaboration of the specific chemical substances by different tissues (chemodifferentiation).

Sometimes we find "differentiation without cleavage." (F. R. Lillie): we have already alluded to this curious phenomenon and seen that the unfertilized eggs of *Chaetopterus* treated with KCl give rise to unicellular ciliated larvae. It must be recognized, however, that these eggs undergo chromosomal divisions and that there may be a synthesis of protoplasm at the expense of the yolk. Differentiation then, is apparently not possible in the complete absence of mitotic activity; however, it can be realized to some extent without the division of the egg into small cells. It is important to determine whether the terms growth and cell division are not synonymous. In the tadpole, for example, the imbibition of water is an important factor in the elongation of the embryo (Davenport, Fauré-Fremiet, and Dragoiu); Glaser has maintained that the increase in hydration plays an essential role in the formation of the nervous system in amphibians, and probably also in gastrulation and in the formation

of the tail bud (Pasteels). But the very precise measurements of density made by Brown, Hamburger, and Schmitt on fragments of the embryo have since established that there are no appreciable changes in water content in different regions of the egg at the gastrula and neurula stages.

Tyler (1939) summarizes the situation as follows: the egg and the embryo can maintain themselves without undergoing growth or differentiation; growth is possible in the absence of the latter but it appears that morphogenesis can not be accomplished without some growth. Let us turn now to the relation between these various processes and the energy resulting from oxidations. Meyerhof and Shearer have concluded from their calorimetric research on the egg of the sea urchin that there is no appreciable energy used in differentiation. Tyler points out, however, that their observations only show that the final energy necessary for morphogenesis is not stored in the egg and that it is dissipated as heat.

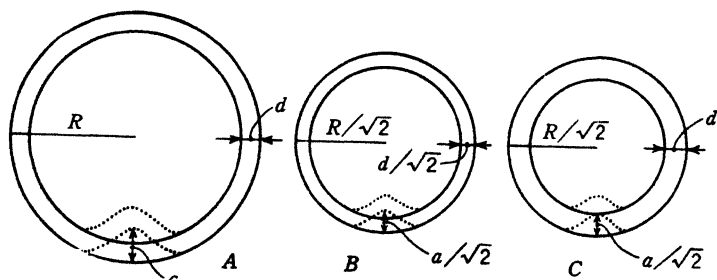


Fig. 58. Normal blastula (A); theoretical model of blastula reduced to half (B); and the actual blastula derived from a half egg (C) (Tyler).

Tyler has approached the question from another angle: He considers, first of all, the case of dwarf embryos obtained by the separation of the first two blastomeres in the sea urchin. We know that they give rise to structurally normal plutei the surface of which is half that of the controls. Since their cells are the same size as those of normal embryos, dwarf blastulae have proportionately thicker outer walls than normal embryos. Figure 58 illustrates this fact: A corresponds to a normal blastula, B to an ideal model reduced by one-half, C to a dwarf blastula such as is actually formed.

We see that if the radius of the normal blastula A is  $R$ , that of the dwarf embryo will be  $R/\sqrt{2}$ ; the thickness of the wall is the

same (d) for A and for C; for the hypothetical model it would be  $d/\sqrt{2}$ . Let us suppose now that to obtain during gastrulation a deformation of the wall to the extent a it would take a force  $F$ ; in the embryo B, the similar deformation  $a/\sqrt{2}$  would necessitate a force  $F/\sqrt{2}$ . But in the case of the actual dwarf embryo C, because of the increased thickness of the wall, it requires a greater force to obtain the deformation  $a/\sqrt{2}$ ; the energy necessary for bringing about this deformation in the normal embryo being  $W$ , that required by the dwarf embryo will be  $W/\sqrt{2}$ . To perform the same amount of work, the pair of dwarf embryos will need a quantity of energy  $2W/\sqrt{2}$  or  $1.41 W$ . The result of these calculations is that the energy necessary for two dwarf embryos to develop from the same egg will be about 40% more than that necessary for a normal embryo.

Following these theoretical considerations, Tyler (1933) compared the respiration of dwarf embryos with that of controls: he found that the rate of oxygen consumption is the same in the two cases but that the development of the dwarf embryos coming from a single blastomere is slowed by 30–40% over that of a normal. This result agrees perfectly with the theoretical concept of Tyler.

The fact that the isolated blastomeres develop more slowly has been known for a long time, but this phenomenon has usually been given an explanation different from that of Tyler. The retardation is supposed to be due to *regulation* which assures the development of the isolated blastomere. One thus assumes that this sort of regeneration of the half-embryo takes some time. This opinion is supported by the observations of Hörstadius and Wolsky who have compared the development of dorsal and ventral blastomeres separated at the 4-cell stage: the former develop more slowly than the latter; and they are also subjected to much more regulation. It is noteworthy that a single blastomere isolated at the 4- or 8-cell stage develops even more slowly. Here again one can not decide with certainty whether the mechanism invoked by Tyler is really in operation or whether the retardation should be considered as a growth regulation. Hörstadius and Wolsky believe that the two factors can coexist and that we ought not *a priori* to exclude either of the two interpretations.

The reverse experiment has been carried out by Tyler (1935). It is evident that giant embryos, obtained by the fusion of two sea urchin eggs should require less energy than the controls in order to reach the same stage. Unfortunately, Tyler did not measure the respiration of the giant embryos which he obtained, since at that

time, techniques of sufficient sensitivity were lacking for the measurements on a small number of cells. In practice, it is possible in the sea urchin to obtain isolated blastomers in abundance, while this is not the case with double eggs. Tyler thus was restricted to showing that the development of giant eggs is faster than normal eggs. This result confirms the findings already obtained by Spemann and Bautzmann in *Triton*.

The absence of any measure of metabolism naturally weakens Tyler's demonstration; it is to be hoped that he will fill in this gap now that the refinements of techniques permit. The problem of the metabolism of half embryos has also been studied by Stefanelli, but unfortunately in a very summary fashion. It is possible to separate the first two blastomeres of the lamprey embryo after a preliminary treatment with sucrose. This sugar, in the concentrations employed, slows development and this requires a supplementary control. The embryos were placed in the sucrose at the two blastomere stage and the oxygen consumption was measured at the gastrula stage. Here are the results obtained:

Normal gastrulae .....	0.0384-0.0396 mm. <sup>3</sup> of O <sub>2</sub> /5 min./egg
Gastrulae treated with sucrose at 2-cell stage (whole) .....	0.0330-0.0342
Gastrulae treated with sucrose at 2-cell stage (twins) .....	0.0342-0.0362

Thus it seems that the respiration of twins would be a little increased by comparison with controls treated with sugar solution; but the measurements are only few in number and have even for Stefanelli only a provisional significance. The experiment deserves to be repeated on a large scale following the metabolism in different stages of development. One can find in this experiment only a slight indication in favor of Tyler's thesis.

The latter has made use of another developmental abnormality (1937). This is the case of sea urchin eggs with *tight membranes*, that is to say, eggs in which the normal elevation of the fertilization membrane is prevented by appropriate treatment. They develop into blastulae with thickened walls and a small segmentation cavity. If the suppositions of Tyler are correct, one should expect that these larvae require a large quantity of energy for gastrulation. This is just what is found. These eggs, like the dwarf embryos, respire at the same rate as controls but their gastrulation is slowed up. They thus absorb more oxygen during this phase of development than

normal embryos. But Tyler himself admits that the morphogenesis of these embryos, especially the migration of mesenchyme cells, is profoundly altered.

The action of temperature provides another possible analytical procedure (Tyler, 1936; Tyler and Humason). One can not, by varying the temperature, dissociate growth and differentiation from simple maintenance, all these processes having the same temperature coefficient ( $Q_{10}$ ) in the species studied (*Dendraster*, sea urchin, *Urechis*, ascidian). The coefficient is also identical for both oxygen consumption and rate of development; furthermore, there is no great difference in the temperature coefficient for the oxidations of unfertilized and fertilized eggs although the former only utilize the energy for maintenance. In the frog, Hoadley has shown that high temperatures stop gastrulation without affecting mitoses; thus a dissociation between growth and differentiation is obtained. This observation agrees well with the fact that the  $Q_{10}$  for the development of the amphibian egg changes during segmentation and later stages (Ryan). However, here too the temperature coefficients for development and for respiration are the same (Altas). The result is that the amount of oxygen absorbed to reach a given stage is the same regardless of the temperature. Let us mention again that, according to Bodine, the temperature coefficients of grasshopper eggs in diapause and in development are much the same; the latter exhibiting only growth and differentiation.

Finally, let us recall the work already mentioned in Chapter IV in relation to the metabolism of the eggs which differentiate without cleavage (J. Brachet; Tyler and Horowitz; Holter and Zeuthen). These workers showed that the oxygen consumption increases less rapidly than in the case of the controls. However, this difference is very small in the case of eggs of *Ciona* studied by Holter and Zeuthen which do not form new cell membranes and where no organs differentiate. This leads one to think that growth, or at least mitosis, is accompanied by the use of energy. It is in the same sense that one interprets the work of Bodine and of his collaborators on the grasshopper egg. Respiration of the egg in diapause, where growth and differentiation are lacking, is considerably lower in comparison with stages during active development. We shall return once more and at greater length to this interesting observation.

Tyler's concept thus rests on an assemblage of coherent facts,

but the character of this evidence is very often indirect. This is why one can not accept as formally demonstrated the idea that growth and differentiation require a relatively important share of the energy. Nevertheless, the ingenious experiments of Tyler appear to us, taken in entirety, more striking than those of his predecessors. The calorimetric findings of Tangl, Meyerhof, and Shearer—in opposition to Tyler's conclusions—rest on a doubtful interpretation because our knowledge of the biochemistry of the egg is still too incomplete for an exact balance of the oxidations. The possible existence of reactions coupled with oxidation-reductions enter to complicate the situation as Rapkine has rightly pointed out.

The experiments of the authors who have tried to measure the work done by the egg during cleavage by determining the *energy of inhibition* appear of still more doubtful significance. They consist in placing sea urchin eggs in hypertonic solutions and establishing an osmotic pressure corresponding to the inhibition of development. One can then calculate the energy necessary for the inhibition of segmentation (Spaulding, Vlès and Dragoiu). An analogous procedure has been used by Fauré-Fremiet, Henri, and Wurmser who have made use of ultraviolet radiation of eggs to determine the energy necessary to stop mitoses. These procedures are open to criticism on several counts. First, segmentation is not a reversible phenomenon; and second it is not proved that the energy of inhibition equals that used for the work of cell division. It is possible, too, that the agents employed, especially ultraviolet, act selectively on an enzyme or a chemical complex necessary for mitoses, the nucleic acids, for example. One should not be surprised, therefore, if different investigators have arrived at values which are extremely divergent.

One attempt of a similar kind made by A. R. Moore in relation to gastrulation is subject to the same difficulties. Moore blocked gastrulation in *Dendroaster* embryos by the addition of sugar. He concludes from his measurements that the forces necessary for gastrulation reach only 3.88 to 7.75 mg./mm.<sup>2</sup> which would expend only 0.05% of the energy furnished by respiratory exchange. It should be noted that Waddington, by a more direct method, arrived at a still lower figure for amphibians. He very ingeniously introduced a small ball of iron into the gastrula and measured the force applied by means of an electromagnet to prevent invagination of the foreign body. He obtained a value of 0.34 mg./mm.<sup>2</sup> Here again

one comes to the conclusions that the movements of cells which characterize gastrulation need but a small amount of energy. The experimental conditions under which Waddington worked however, are not exactly stated in his short note; therefore, we can not at present draw any certain conclusion from his experiments because of the great complexity which attends gastrulation in the amphibians. In addition, Holtfreter has drawn attention to a possible source of error in Waddington's experiments: the gastrula cells adhere very closely to instruments made of iron, a fact which may modify completely the results of the experiment.

Thus it is a future problem to reconcile, if possible, the contradictory results of Tyler and those of Moore and of Waddington. What is quite certain is that the respiratory exchanges increase during the course of development and it is obviously tempting to relate this increase with progressive morphogenesis, such as Tyler has done.

Let us look into some of the recent work devoted to the study of respiration during development, beginning with those concerning the amphibians. Increase in oxidation here is considerable, since the tadpole at hatching consumes 10 to 15 times more oxygen than the fertilized egg. It has often been discussed whether this increase is continuous or occurs stepwise. According to Bialaszewicz and Bledowski, the gaseous exchange increases very regularly as a function of time, following the relation:

$$y - a = kt^2$$

where  $y$  = respiration of any stage,  $a$  = the respiration of the unfertilized egg,  $k$  = a constant, and  $t$  = time. On the other hand, Parnas and Krasinka have maintained that respiration does not vary during cleavage and shows abrupt changes at gastrulation, and again at the time of the closure of the neural folds. They concluded from this that morphogenesis, properly speaking, requires energy while the division into smaller and smaller cells does not need it. This conclusion, as Brachet showed in 1934, is not correct because it rests on measurements made at spaced intervals. When one follows the oxygen consumption continuously one finds an increase in oxidations beginning during segmentation; following this the curve rises more and more.

The more recent works of Wells, Fischer and Hartwig, Atlas, Stefanelli, Barnes, Moog, Steinbach and Spiegelman, and Boell have

confirmed the fact that the gradual rise in respiration follows a parabolic curve; Figure 59, taken from Atlas, illustrates this fact. All the recent authors have confirmed an observation first made by Atlas; that is, that the curve shows a discontinuity at the time when gastrulation ends and the embryo begins to elongate. According to Barnes, a first discontinuity is found at the beginning of gastrulation agreeing with the earlier results of Parnas and Krasinska; but Moog, Steinbach and Spiegelman, and Boell do not appear to have confirmed this phenomenon.

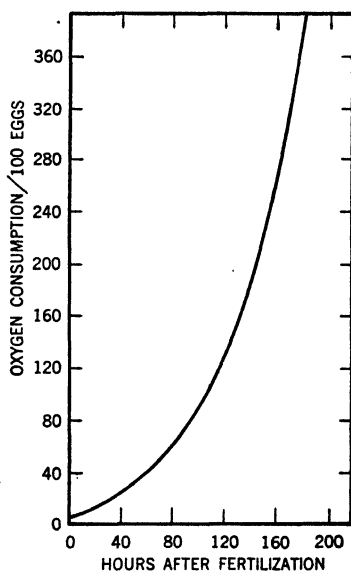


Fig. 59. Oxygen consumption of frog egg as a function of time (Atlas).

Wills' measurements, which have been carried out through a very advanced stage, show that the oxygen consumption expressed in  $\text{mm}^3$  per mg. nitrogen increases at first following a sigmoid curve; it attains its maximum during metamorphosis. As to later stages, the young frog respires at a lower rate at equal weight than the tadpole.

Fischer and Hartwig have drawn attention to another aspect of the problem. Observing distinct differences in the respiration of various amphibians, they wondered if there was a relation to the weight or to the surface of the egg. Comparing the gastrula of *Triton* and that of Axolotl, they found an oxygen consumption 2.2



times higher for embryos of the second species. If one relates the values to the same weight one finds that the *Triton* gastrula, with less weight, respire proportionately more than that of *Axolotl*; the figures are, respectively, 0.11 and 0.17 mm<sup>3</sup> of O<sub>2</sub> per milligram of dry weight per hour. If, on the contrary, one relates the figures for respiration to the surface the agreement is perfect. Thus it is the surface which is important in the gaseous exchange. Let us add that the curve obtained by Fischer and Hartwig follows approximately the equation of Bialaszewicz and Bledowski.

However, Atlas does not admit the validity of this formula. His curve corresponds better to the relation:

$$y = ae^{kt}$$

where  $y$ ,  $a$ ,  $k$ , and  $t$  have the same significance as in the formula of Bialaszewicz and Bledowski. Nevertheless, satisfactory results are obtained only if the value of the constant  $k$  is changed at neurulation. The curve in reality shows a slight discontinuity at this time. Atlas relates this fact with the beginning of the growth in length which is exhibited at this time; he thinks that the increase in respiratory rate represents a constant percentage of the growth in length at neurulation. This elongation is, as we know, in direct proportion to the increase in water imbibition. Atlas has confirmed this fact in determining that growth in length precedes somewhat the absorption of water.

According to Atlas, respiration does not increase in a manner directly proportional to the volume of the embryo; further if we compare two different species (*Rana pipiens* and *Rana sylvatica*), we see that larvae of the same length respire with the same intensity. The tadpoles of the first species are, however, richer in water. Atlas is of the opinion that growth of the tail by cell division is the principal cause of the increase in the oxidations after neurulation. This increase is more the result of a multiplication of the number of cells than an increase in the respiration of each individual cell. However, recall that Pasteels, basing his statement on numerous measurements of the mitotic index, denies categorically that growth of the tail bud is a result of mitosis. Likewise, according to Gillette, cellular division during neurulation remains moderate (23%) in the ectoderm. It is seen that the problem is in reality very complex.

It has also been attacked by Spirito and by Stefanelli (1938). Spirito compared, like Fischer and Hartwig, the respiration of the

eggs of different related species (toads, *Discoglossus*, frogs, *Hyla*, axolotl). Naturally he found great variation from one case to another. Certain eggs, such as those of *Discoglossus* and of axolotl, have a high respiration while the oxygen consumption is particularly low in *Rana agilis* and in *Hyla*. These differences persist even if one relates all these figures to the diameter of the egg by making a simplification of the shape of the egg; but Spirito finds that the amount of oxygen absorbed in passing through the same stage of development—neurulation, for example—is remarkably constant in all the eggs studied. We fear, however, that the exact duration of neurulation can not be determined with accuracy, since this phenomenon presents too great a variability from one species to the next.

As to Stefanelli, he has come to conclusions resembling those of Atlas. He has not found a direct relation between the oxygen consumption and the time elapsed since fertilization nor with the size of the embryo; but he finds, like Atlas, a change in the rhythm of the oxidations at neurulation when the spherical larva begins to elongate. Stefanelli correlates this fact with the increase in surface, with the utilization of the yolk, and with the growth of cells between each mitosis which occurs at this time. It is true that yolk utilization accelerates during neurulation although it has begun earlier; the point as to whether the number and the size of the cells increases abruptly at neurulation does not appear to be clearly established and one wishes that Atlas and Stefanelli would test their hypothesis by means of cell counts. Thus we see that no truly satisfactory explanation has yet been furnished for the discontinuity in the curve for oxygen consumption first reported by Atlas.

Stefanelli has also compared eggs of *different size* in the same *species*. Their development is synchronous but they have different respirations. However, if one relates the figures to units of mass, it is found that the same quantity of embryos absorbs the same number of cubic millimeters of oxygen in the same time or to attain the same stage.

If, on the contrary, one compares the *eggs of different species* (however closely related) we find that the rate of development varies. Equal units of mass of these embryos will absorb the same amount of oxygen to pass through a definite stage of development in conformity with Spirito's results, but they show varying oxygen consumption during equal times.

Summarizing, the oxygen consumption of batrachian eggs rises in a progressive fashion, showing an acceleration at the time when the embryo begins to elongate. The time relations between this curve and the length and surface of the larva are too complex for any simple analysis. Similar results are found with other forms. In the sea urchin, respiration increases during cleavage but without any simple relation to the number of nuclei (Warburg, Meyerhof, Gray, Rapkine, Ephrussi, etc.). According to Gray, the curve rises, as in amphibians, following an exponential function with time. This opinion has been confirmed by Lindahl and Öhman (1938) and by Lindahl (1939). Oxygen consumption follows first an S-shaped curve, which flattens off when the blastula emerges (Fig. 60).

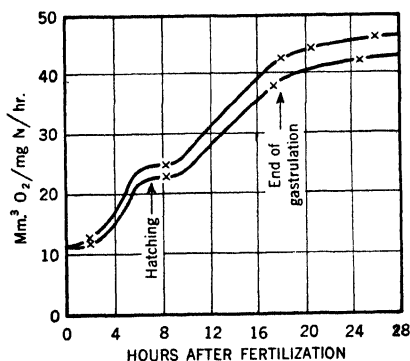


Fig. 60. Oxygen consumption of sea urchin egg from fertilization to hatching (Lindahl).

One sees that development is subdivided into three periods in relation to oxidations. The first phase, about the first 9 hours, is characterized by an S-shaped curve. Between 9 and 18 hours gastrulation progresses: this is the "period of the formation of the mesentoderm" (Lindahl). The third period corresponds to the differentiation of the gastrula into the pluteus. It is interesting that the R.Q. differs in the first two phases (Öhman). It is only 0.73 immediately after fertilization and changes to 0.85 at the end of 7-8 hours. One can conclude that the egg, when cleaving, oxidizes chiefly fats or proteins, while the utilization of carbohydrates begins during the formation of the mesentoderm. This is in agreement with the direct determinations of Clowes and Krahle who found a preponderance of protein oxidation during cleavage followed by carbohydrate oxidation. More pronounced qualitative differences have been found in the frog: Brachet has found an R.Q. of 0.65 during cleavage and of 0.9-1.0 at gastrulation.

Numerous investigators have noted an increase in the oxygen consumption during embryonic development of widely different organisms: Meyerhof, Buglia, and Baldwin in mollusks; Fink, Bodine, and Wolsky in insects; Philips in *Fundulus*; Bohr and Hasselbalch, Murray, and J. Needham in chick. The last case has been discussed by Needham at some length in his book. Recently this subject has been re-examined by Philips who used modern methods permitting measurements at the very early stages of development. The oxygen consumption per embryo doubles during the first four hours of incubation. However the  $Q_{O_2}$  ( $\text{mm}^3$  of  $O_2$  per mg. dry weight or per mg. of N per hour) does not change during the first three days; its value is about that characteristic of most of adult mammalian organs. The increased respiration of this embryo is thus coupled with growth of the blastodisk and cellular multiplication. This conclusion agrees perfectly with those adopted by Atlas and Stefanelli in the amphibians, in the sense that oxidations increase because the number of cells augments and not because the respiration of each individual cell increases.

In the rat, Boell and Nicholas followed oxygen consumption using the Cartesian diver and found that respiration increases from  $0.0007 \text{ mm}^3$  of oxygen per egg per hour to  $0.01 \text{ mm}^3$  during cleavage. It attains a value of  $0.2 \text{ mm}^3$  in a ten-day embryo. But if one determines the  $Q_{O_2}$  and, as a result, relates the values to the *weight* of the embryo, one sees, on the contrary, a drop from 30 to 12–15, comparing the fertilized egg and the embryo with a few somites. It must be admitted that in this case the respiration of the cells taken individually probably decreases. It would be interesting, although difficult, to determine how oxygen consumption in the frog changes in the various cells by making a correction for the presence of the yolk; development is probably accompanied by a synthesis of "pure" protoplasm and it may be that the latter obeys the same laws as the embryos of the hen and the mammals in regard to oxidations. Unfortunately, it is not easy to determine the volume occupied by the cytoplasm and yolk, respectively; the method of centrifugation used by Conklin for this problem has been the object of criticism, justified in the main, by LeBreton and Schaeffer.

We have thus far presented chiefly the quantitative side of the problem. Are there any reasons for thinking that the embryo utilizes the reserve materials at its disposal in any regular order? In other terms, do we have, as Needham thinks, a *succession of energy*

sources? According to Needham, the embryo uses first carbohydrates, then proteins and fats. From this there results a lowering of the R.Q., obtained in a number of cases: chick (J. Needham 1931, 1932; Dickens and Simer), *Fundulus* (Amberson and Armstrong), grasshopper (Boell), crab (Needham, 1933) *Limnea* (Baldwin). Opposed to these results are the observations of Öhman and those of Clowes and Krahle on the sea urchin egg and of Brachet on the frog's egg, but it is important to note that the low R.Q. observed in these two cases is only found during cleavage. As soon as this is over the R.Q. increases and it may very well be that it drops later when the glycogen reserve is expended. Thus there would be an oxidation of substances other than carbohydrate in the sea urchin and the frog up to the blastula stage; at this time the metabolism of sugars would begin, followed by the use of other sources of energy in the order claimed by Needham. This opinion receives reinforcement from the fact that the use of fats in the tadpole certainly occurs much later than that of carbohydrates (Bialaszewicz and Mincovna; Atlas). Determinations of carbohydrate in the grasshopper embryos made by Hill confirm Needham's theory in this material. It is in the beginning of development when carbohydrate metabolism is most intense. Obviously one must not take this rule established by Needham too literally. Probably the embryo oxidizes simultaneously the reserve materials at its disposal, but one among them is more likely used to a greater extent at certain stages of development. In fact we know that the frog egg excretes urea and ammonia during cleavage, but that nitrogen excretion increases considerably in the tadpole (Bialaszewicz and Mincovna; J. Brachet, 1939). It is well to note again that the interpretation of the R.Q. is often erroneous when it is not supported by determinations of the carbohydrates, fats and proteins. The figure obtained is in reality only an average of the oxidations within the cell; the presence of oxidation-reduction or of auto-oxidation can greatly influence the result. In spite of these reservations, however, we may consider that Needham's theory contains a great deal of truth.

Up to the present, we have talked only of oxidation; it should not be forgotten that fermentation—*glycolysis* in particular—also furnishes much energy. Recall that glycolysis is usually influenced by respiration, the latter tending to inhibit lactic acid production according to the Pasteur-Meyerhof reaction. The intimate mechan-

ism of this reaction is not entirely clear. The question of the respective roles of respiration and of glycolysis during growth and differentiation has been raised by the famous work of Warburg on the metabolism of embryonic and cancer cells. In the mammalian embryo the intensity of glycolysis decreases during development; thus Warburg thought there could be no growth without glycolysis.

Brachet, as well as Lennerstrand, has shown (1934) that in the frog egg cleavage is possible under anaerobic conditions without the formation of important quantities of lactic acid. Thus there is not necessarily an increased glycolysis when mitotic activity is high. But we hasten to add that cleavage is not accompanied by growth. If glycolysis is necessary for the former we may infer that this reaction is linked with the increase in size of the cells between two divisions. This explanation is the more plausible since the tadpole, which undergoes considerable growth, has all the characteristics of a preponderantly carbohydrate metabolism: R.Q. about 1, strong glycogenolysis, and increased production of lactic acid under anaerobic conditions. These characteristics are lacking in the morula.

A study by Demuth has dealt with the metabolism of advanced tadpoles up to the point where they metamorphose. On studying the effects of specific inhibitors of respiration and glycolysis, the author believes that differentiation is coupled with the former and growth with the latter. Unfortunately, Demuth did not control the influence of the agents which he used on metabolism. His material, which is very complex, is thus not very favorable so that his conclusions do not carry conviction.

For this reason the question of the relations of growth and differentiation to glycolysis and with respiration has been re-examined by Trifonova and her collaborators. The principal work, 1937, was carried out on the egg of the perch. The author divides development into a series of phases: first cleavage, characterized by division without growth; gastrulation, which is accompanied by considerable growth, followed by a period of differentiation of the axial organs; and finally the second phase of growth corresponding to the elongation of the embryo.

Cleavage is paralleled by acidification of the cytoplasm which stops at gastrulation; the period of differentiation that follows coincides with an increase in the pH of the embryo. Then, during the second period of growth, the pH tends to drop again. Unfortunately

the measurements were made by means of vital staining with neutral red and this method, as we know, is subject to several reservations.

Direct determinations of oxygen consumption and of lactic acid production are more demonstrative. Respiration increases during cleavage and tends to drop off during gastrulation; it increases at the time of the formation of the axial organs and decreases again when the embryo elongates. Thus, the periods of growth are distinguished

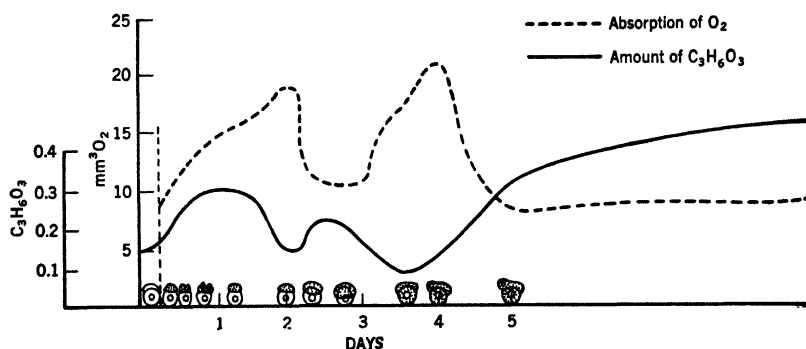


Fig. 61. Oxygen consumption and glycolysis during development of the perch (Trifonova).

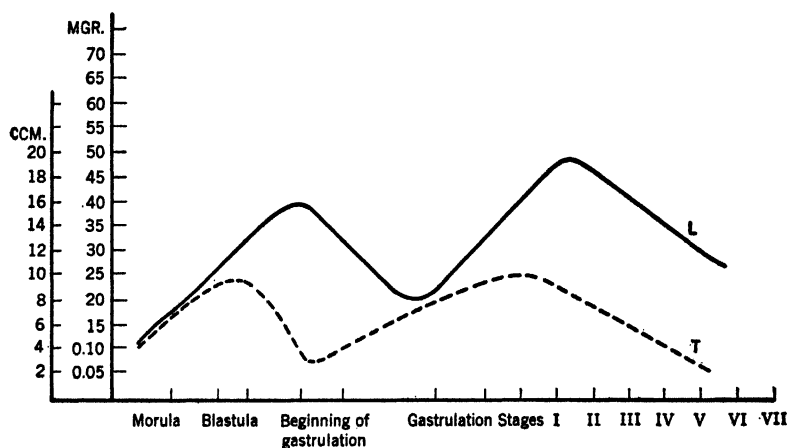


Fig. 62. Aerobic glycolysis in mg (broken line) and anaerobic CO<sub>2</sub> production (solid line) in mm<sup>3</sup> per 100 eggs of the frog. (T = Trifonova, Korovina, and Sliusarev; L = Latinik-Vetulani).

by a low respiration, those of differentiation by a high oxidation. The determinations of lactic acid form a curve exactly the inverse (Fig. 61). Glycolysis is intense during the phases of growth and becomes lower when differentiation is going on. Trifonova concludes from this that the equilibrium of the Pasteur-Meyerhof reaction is displaced in a cyclic fashion during development. Conforming to Demuth's ideas, oxidations predominate during differentiation, while glycolysis is more important in growth. Almost identical results have been obtained by Trifonova and her collaborators in the eggs of the salmon and the frog. In the latter (Fig. 62), glycolysis increases during cleavage, decreases at the beginning of gastrulation, and rises once more during this latter process; a second drop in glycolysis characterizes neurulation.

The cyclic nature of glycolysis has not been found by previous investigators who studied the frog's egg (Lennerstrand, J. Brachet, J. Brachet and J. Needham). They observed only a progressive increase in glycolysis and in glycogenolysis, but it may be that their measurements were not made at frequent enough intervals to reveal the differences described by Trifonova, Korovina and Sliussarev. It must be added, however, that the determinations made by Latinik-Vetulani on the production of  $\text{CO}_2$  in anaerobiosis (Fig. 62) do not agree perfectly with those of Trifonova and collaborators. Nevertheless, they are an index of carbohydrate metabolism in the absence of oxygen; it is weak during cleavage and increases at the beginning of gastrulation. The anaerobic  $\text{CO}_2$  production decreases during gastrulation, rises again at the beginning of neurulation, and declines once more during this phase of development. Curve L of Figure 62 corresponds to the figures of Latinik-Vetulani which can be compared with the values established by Trifonova, Korovina and Sliussarev (curve T).

If one attempts to evaluate the results of these authors, one must confess that the demonstration, at least for the amphibians, is not absolutely satisfactory, since we know that respiration increases progressively up to neurulation. At this time a growth phase begins which is characterized by an increased respiration (Atlas, Stefanelli), although one ought to find the opposite effect. We also note that it is difficult to decide whether gastrulation, for example, is a true growth phase; this interpretation is very improbable in the amphibians and the fishes (Pasteels). Finally, other complications



arise from the fact that growth of the tadpole is largely a matter of imbibition of water. The hypothesis of Trifonova is certainly interesting and justified, but one would prefer a better demonstration. It would be especially desirable to compare the respiration and glycolysis of normal embryos and anidic ones, since the latter show growth without differentiation.

Let us try to derive some *conclusions* from this assemblage of facts. Development is undoubtedly accompanied by a progressive increase in metabolism. It appears logical to suppose as Tyler does, that a part of the energy furnished by oxidation serves to insure growth and differentiation, although certain findings lead one to believe that the process does not need much energy. Cleavage is characterized, at least in the sea urchin and frog eggs, by a special metabolism from a qualitative point of view as indicated by the low R.Q. The coupling generally established between growth and carbohydrate metabolism does thus not necessarily hold during cleavage. One deduces that the utilization of sugars is more in relation to the synthesis of protoplasm between mitoses than with cell division, properly speaking. Needham's rule relative to the succession of energy sources seems to have general application except for cleavage. The embryo first uses carbohydrates and does not attack the proteins and lipids in appreciable quantities until later. Finally, there appears to be some relationship between growth and glycolysis on the one hand and differentiation and respiration on the other.

From a very general point of view one gathers the impression that there is no very direct coupling between oxidation in the egg and its morphogenesis. None of the facts discussed above, interesting though they are, contribute much to an explanation of the chemical basis of embryonic development; nothing we have found indicates that respiratory metabolism is the cause of embryogenesis. All that we can say is that the latter needs energy to be completed. It is important to know whether carbohydrate metabolism predominates at the time when the egg is undergoing its primary morphogenesis; the utilization of carbohydrates, however, is not sufficient to distinguish the egg from another cell with an entirely different function as, for example, a muscle cell. In fact, the embryologist finds himself in almost the same situation as the physiologists interested in the basic mechanism of muscular contraction. Numerous

works have led to an admirable analysis of the carbohydrate metabolism of muscle without helping us much to understand the manner in which contraction takes place. It is clear that recent research on the properties of muscle proteins has revealed to us new and precise horizons and it is to be hoped that it will be the same for the developing egg. But we must not underestimate the interest of the facts acquired about the metabolism of egg and muscle; we shall not really understand the basis of embryonic development nor that of muscular contraction until we have bridged the gap between the metabolic findings and those relative to the structure of the proteins. In this regard, the work of Engelhardt and of Szent-Györgyi and his school, demonstrating the direct relation between the contraction of fibers of myosin and the breakdown of adenosine triphosphate, constitutes a model that may serve as an inspiration for chemical embryology.



## CHAPTER VIII

# Chemical Embryology of the Invertebrates

### 1. Mosaic and Regulative Eggs

We have already pointed out that for a long time experimental embryology has distinguished two different types of eggs, those composed of a *mosaic* of organ-forming areas and those capable of *regulation*. Let us review in a few words the reason for this distinction. When the first two blastomeres of the sea urchin egg are separated, we find that each of them gives rise to normally formed embryo the size of which is reduced to one half (Driesch). Had there been no experimental manipulation each of these blastomeres would have produced only half an embryo; thus the isolation of blastomeres reveals new potentialities resulting in the phenomenon of regulation. This latter really corresponds, in the particular case of the egg cell, to a regeneration of a missing part. But the result obtained in the sea urchin is not a general one. In a number of invertebrates, the egg is unable to perfectly repair the defect and it develops into an abnormal larva in which certain organs are more or less completely lacking. Thus we consider this egg-type as a mosaic of areas destined to give rise to definite portions of the larva. These regions are the *germinal localizations* which become apparent even in an early stage of ontogenesis.

Recent research has somewhat modified this concept. In fact, it has been shown that all eggs are capable of a certain degree of regulation which is quite variable; considerable in the sea urchin egg, regulation is much more limited in the eggs of ascidians, and in those

of a number of worms and mollusks. The fact that even the mosaic type of egg exhibits a certain amount of regulation indicates that the former distinction was too sharp. For more details on this subject we recommend to the reader the book by A. Dalcq (1941) in which the question of regulation is treated with thorough competence. The author rightly points out how unfounded the distinction is between mosaic and regulatory eggs in certain cases. We know that one of the first two blastomeres of the egg of *Triton* will give a complete embryo (Herlitzka); on the contrary, in the frog egg after the destruction of one blastomere the other forms only a half embryo (W. Roux, A. Brachet). From this one might deduce that the *Triton* egg exhibits regulation while that of the frog is a mosaic of regions; but we now know that the anurans and the urodeles behave almost identically with respect to the fundamental mechanisms of morphogenesis: structural outlines, mode of gastrulation, inductors, etc.

If we limit ourselves to the invertebrates, we see that, depending on the species, the "mosaic" or "regulative" character is exhibited more or less decisively. Without diametrically opposing these two types of eggs we may, nevertheless, expect a more marked heterogeneity of the cytoplasm in those species where germinal localizations are more precise. Thus one must foresee the existence of all possible intermediate conditions between the two extreme types. Furthermore it is only for the sake of simplification that we will use the terms "regulation" and "mosaic" for egg-types and we will keep in mind the reservations just made.

A number of investigators have attempted by cytochemical methods to throw some light on the differences between forms whose eggs tend to be regulative and those which are mosaic.

We will review the chief results acquired and consider separately the egg of the sea urchin which has been the object of a particularly exhaustive analysis. However, one last preliminary remark is called for. That is, attempts to prove the existence of distinct regions in mosaic eggs were made before the adoption of cytochemical methods; cytological examinations (E. B. Wilson) and sometimes even the study of the pigmentation of the living egg have often furnished valuable information, and in this field the classical work of Conklin on the egg of the ascidian *Styela* comes to mind. Close examination of the pigments led him to distinguish a series of "plasms" or organizing regions and to follow their behavior during maturation and

fertilization. The use of cytochemical techniques has the great advantage of bringing us information on the chemical nature of these primordial plasms.

The first sustained analysis of the changes in the egg following fertilization and leading to the segregation of germinal localizations was made by Spek (1930). Working on the egg of the polychaete worm, *Nereis dumerilii*, he used the ultramicroscope and vital staining for the most part. With these simple techniques he saw that during oögenesis a citron yellow pigment is formed in the cytoplasm; in an acid medium this pigment changes to violet. After fertilization, the egg took on a brown tint, while the cortex broke down to give rise to the jelly. The brown coloration results from the hydration of the hyaloplasm, which shows changes in its refringence. After the extrusion of the polar bodies one witnesses a migration of the yolk, the fatty inclusions, and the yellow pigment toward the vegetal pole, the animal pole being now clear and free of particles. This separation of the egg materials into layers (*bipolar differentiation*) is maintained during cleavage; however, at about the four blastomere stage one notices a change in the pigment toward a violet color. This is probably due to an acidification of the vegetal pole material, since a change in the direction of acidity is observed when the egg is previously stained with neutral red or Nile blue sulfate. The use of these indicators proves that the animal pole simultaneously becomes more alkaline. During further development, the acid-staining cells give rise to the endoderm while the alkaline-staining blastomeres form the ectoderm.

Since the separation of acid and alkaline materials after maturation cannot be attributed to gravity, Spek favors the hypothesis of a migration by cataphoresis of the colloids of the egg toward the two poles. Thus the  $H^+$  ions would accumulate at the vegetal pole and the  $OH^-$  ions at the animal pole.

As a matter of fact these observations by Spek are similar to some earlier ones by G. Teissier (1925) who drew attention to the change in coloration produced during development in the hydroid, *Clava squamata*. These eggs contain a brown pigment which changes to orange during gastrulation, the change beginning in the ectoderm and at one pole of the planula larva. Since it coincides with the differentiation of the cells and the utilization of the yolk, Teissier considered this pigment, apparently a lipoprotein, to be sig-

nificant only as a waste substance. Pigments of this type are indeed very common in the hydroids. The originality of Spek's work rests chiefly on the interpretation which he made of the change observed in *Nereis*. He saw the significance of the acidification and hypothesized a migration of colloids of opposite charges to the two poles.

Spek verified the phenomenon of *bipolar differentiation* following maturation in *Chaetopterus* and *Nereis limbata*. The use of vital dyes to measure the pH gives a value of 7.8 for the animal pole and a low figure for the opposite pole. Spek's figures indicate a very clear difference between the alkaline ectoderm and the acid endoderm at gastrulation. Later on, a new differentiation is seen in the ectoderm itself. The cells outside have a more alkaline reaction than those in the interior. Furthermore, bipolar differentiation is not an exclusive property of worm eggs. Spek has reported it in cephalopods (1934) and in teleost fishes (1933). In both cases, he took care to control the results of vital staining by using another method. He injected indicators that do not penetrate readily and he even used these dyes on droplets taken from the two halves of the egg. According to Spek (1938), the localizations described by Conklin in the *Styela* egg would be the result of a bipolar differentiation. Here again the alkaline substances migrate to the animal zone while the vegetal pole gives an acid reaction. Even in the guinea pig, Arnold has detected a bipolar differentiation of vitally stained embryos; it manifests itself very late however. In addition, Spek has observed a very clear bipolar differentiation in the larvae of sponges.

Spek's results have been confirmed by Dorfman's work, which utilized quite a different technique and other material. When micro-electrodes were introduced into frog eggs, a more acid reaction was observed in the vegetal pole, and this is also the case in the oöcyte before maturation.

Although Spek's results are received without question, the interpretation he offers has raised a number of criticisms. Teissier (1935) studied bipolar differentiation in the egg of *Pleurobranchia pileus*, which shows the phenomenon with great clarity; he found the difference in color of the indicators used by Spek to be retained even after the fixation of the egg with formalin or with  $\text{HgCl}_2$ . The use of pH indicators such as phenol red, which is unquestionably superior to vital dyes, shows no variations in pH in the egg. Teissier rightly points out that it is difficult to see how such a difference

could be maintained after fixation and also that it can not be demonstrated with the best indicators, and concludes that the vital dyes used must change color under influences other than pH.

A very plausible explanation of Teissier's results has been suggested by Lison. He has shown that vital dyes exhibit, besides an ionic color change, a *metachromatic* effect; for example, adding a trace of a sulfuric ester will produce a change in color. These observations, proving that it is possible to detect traces of sulfuric esters by the metachromatic reaction, lead to the conclusion that vital dyes can not serve to measure the intracellular pH. Lison emphasizes that his criticisms do not apply to those cases where Spek has made use of dyes of proven value and that they do not change Spek's theory in themselves but simply sound a word of caution about the significance of certain results. When Spek finds a pH of the order of 10, it is probably a metachromatic color change due to the presence of sulfuric esters, for the metachromatic error can attain as much as six pH units.

Spek has devoted a long paper to the refutation of these objections. Since a discussion of this paper would be too time-consuming, we simply point out that the metachromatic reaction does not have the absolute specificity claimed by Lison, although the sulfuric esters are exceptionally active in this respect. Further, certain of Spek's experiments tend to show that the presence of these esters does not affect the change in the indicators when one uses them as vital dyes. However, P. Dustin has recently established that the vital dyes in the cellular vacuoles reach a sufficient concentration to show the metachromatic change; he concludes that all measurements of pH with basic dyes remain open to criticisms.

It seems opportune here to remember that investigators who have measured the pH of eggs by microinjection of dyes which show no metachromatic effect never have observed differences between the two poles of the cell (J. and D. Needham; Chambers). These negative results were obtained for the sea urchin egg and in *Fundulus*. One would expect that the latter, at least, would conform to Spek's rules. Spek has attempted to interpret Chamber's observations by assuming that microinjection allows only the measurement of the dispersion medium while the vital dyes become fixed to the granules of the dispersed phase. If this explanation is correct, bipolar differentiation pertains exclusively to the inclusions of the egg without



affecting the hyaloplasm. However, it is to the latter that embryologists assign the principal role, in view of the centrifugation experiments of Conklin and the more recent ones of E. B. Harvey and Clement. The last two investigators have obtained normal development from fragments of the centrifuged egg which contain only hyaloplasm (*Arbacia*, *Physa*). This opinion is not universally accepted (Raven), however, and it is probable that the morphological importance of the granules varies from one species to another.

Let us add a word concerning the criticisms which Reiss (1936) has leveled at Spek's theory. He strongly emphasizes the fact that the pH has significance only when it is concerned with the dispersion medium. The significance of the changes observed by Spek in the case of interfaces or of the dispersed phase is necessarily obscure: the isoelectric point of the proteins and the presence of lipids in the vacuoles which are vitally stained affect the results. Adsorption phenomena can also influence the color.

It is seen, then, that the methods used by Spek most frequently are very suspect and that one can not draw hard and fast conclusions from them. Furthermore, even the investigators who have applied the above methods do not share Spek's opinions. Thus, Gersch and Ries (1936, 1937), and later Gersch (1939) studied various mosaic and regulative eggs by means of vital dyes which are also pH indicators, and came to the conclusion that bipolar differentiation may be present in certain forms of this second type while it is lacking in some mosaic eggs. Although *Aplysia* behaves like *Nereis*, other mosaic eggs do not. Gersch and Ries reject the hypothesis of a cataphoretic migration. According to them, bipolar differentiation is the result of a separation of the yolk and the "formative cytoplasm." Every time that inclusions become isolated from the hyaloplasm, a bipolar differentiation can be detected by pH vital dyes. Gersch has drawn attention to the fact that the material which gives an alkaline reaction in the living state becomes basophilic after fixation. Conversely the vegetal half, apparently acid in living condition, is acidophilic in histological sections. It must be admitted that basophilia results from the acidity of structures and acidophilia from their alkalinity. The results of vital staining and of histological techniques are thus in contradiction and Gersch wisely concludes that it is impossible to speak of the pH of different regions of the egg because of the excessive errors inherent in the techniques (salt, protein, and metachromatic errors).

A similar point of view has been adopted by Raven in an extensive and well conceived investigation. First of all he points out that the differences between Spek and Gersch and Ries do not bear on the existence of bipolar differentiation but on its nature: whether it is a cataphoretic migration of the colloids of the hyaloplasm or a separation of the yolk which is always moved to one of the poles. To answer this question Raven combined vital staining with centrifugation in *Nereis*, *Chaetopterus*, and *Aplysia*; he made it clear, first of all, that he could not tell if the color changes were really due to variations in pH, so he made use of the terms "acid" and "alkaline" only to avoid circumlocutions. Raven's experiments establish clearly that when the yolk is thrown down by the centrifuge it takes on an acid color. The deutoplasm thus exhibits an "acid" reaction even before bipolar differentiation has taken place. "Alkaline" substances accumulate at the centripetal pole of the centrifuged egg under the fatty cap, but it is not possible to measure the reaction of the hyaloplasm itself, since it does not stain with enough intensity.

The egg can be centrifuged so that the sedimentation of substances takes place at some angle to the normal polarity of the egg, thus establishing that it is sedimentation alone that determines the behavior of the egg toward the vital dyes used. The primary physiological axis of the egg, then, does not influence the results. Bipolar differentiation is never observed in the hyaloplasm: thus there is no reason to believe, as Spek does, that a separation of the colloids of opposite charges takes place in the hyaloplasm. Thus Raven has come to share the view of Gersch and Ries, that it is the displacement of the inclusions, by whatever means, that causes bipolar differentiation. However, this phenomenon has a real morphological significance to Raven, for, contrary to general opinion, he attributes an important role to the microscopically visible inclusions in the eggs of mollusks and polychaetes. As a matter of fact these eggs when centrifuged give rise to very abnormal larvae which show an aberrant distribution of the "acid" and "alkaline" substances. Larvae are never found with an intestine of alkaline cells or a ciliated epithelium with an acid reaction, so it must be concluded that the "acid" yolk is necessary for the differentiation of the intestinal endothelium while an "alkaline" substance is needed for the formation of ciliated epithelium.

These findings agree with those of Reverberi (1940) on the mosaic eggs of ascidians. If the unfertilized egg is cut in two by

ultracentrifugation according to E. B. Harvey's method, the light fragment does not divide as we have seen earlier; it contains the nucleus of the egg, but it lacks a centrifugable substance necessary for division. The heavy fragment gives rise to ectoderm, endoderm, and muscle, but forms neither notochord nor nervous system. Thus these latter structures appear only when the egg contains the substances present in the hyaloplasm. In this connection it should be remembered that the same experiment was carried out by E. B. Harvey on the sea urchin egg, but here both the heavy and the light fragments give apparently normal larvae. It is interesting that the same investigator obtained the same result (1946) with fragments completely free of visible granules.

Since 1938, the question of bipolar differentiation has made little progress, the debate of Spek and Ries turning into a long polemic which we do not wish to discuss here. We gather from this discussion only that the separation of the yolk from the hyaloplasm could result from an internal cataphoretic migration. Nevertheless it will be necessary to submit this hypothesis to the test of experiment, since other factors may intervene, such as the changes in the sub-microscopic structure of the cytoplasm (see Frey-Wyssling).

But more recently, Costello (1945) proposed a new physical explanation of bipolar differentiation to which he attaches a morphological significance. The phenomenon may result from the building up of a diffusion potential (Theorell effect) as a result of the constant penetration of some substance at a certain region of the egg (for example, the animal pole). Under these conditions, diffusion potentials may be set up in certain regions of the protoplasm, which will then function like a multiple membrane. All electrically charged particles, regardless of their dimensions, will be displaced in this diffusion potential field. The diffusible substance could merely be one of the ordinary ions in sea water. This interesting explanation is as yet only a hypothesis that, however, has the advantage of not presupposing a flow of electric current through the cell.

Let us conclude by saying that maturation is often accompanied by a migration of various materials toward the two poles of the egg, resulting in an accumulation of yolk at the vegetal pole. This movement is also shown by vital staining, the yolk behaving as if it had an acid reaction, while the animal pole appears alkaline. The question as to whether these differences are really a matter of pH remains

controversial. The phenomenon discovered by Spek does not pertain specifically to mosaic eggs since it is absent in some of them and present in forms capable of considerable regulation. Nevertheless, bipolar differentiation appears to be an important morphogenetic factor and it is to be hoped that future studies will define the chemical nature of the "acid" and "alkaline" constituents, as well as the reasons for their separation at the time of maturation.

In view of the work of Ries, this analysis can be said to have already begun: this investigator has compared mosaic and regulative eggs from different viewpoints; by means of cytochemical methods he has studied especially the behavior of ascorbic acid (vitamin C), glutathione, the oxidases, and  $rH_2$ . Let us review the main results obtained.

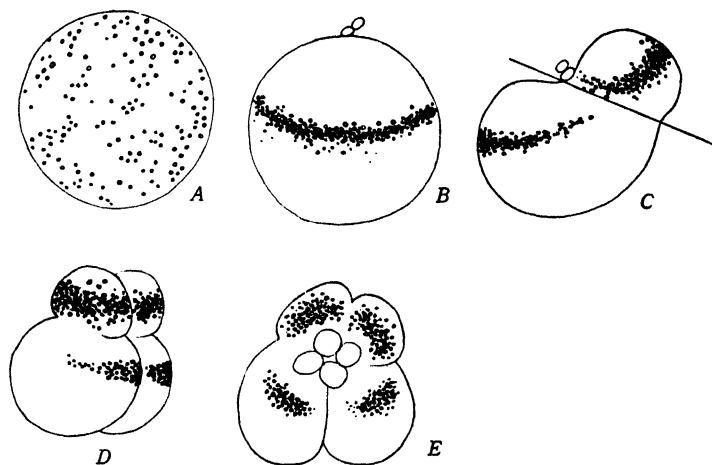


Fig. 63. Localization of ascorbic acid in *Aplysia* (Ries).

In his important work of 1937, Ries studied principally *Aplysia*, the sea urchin, the ascidian *Ciona*, one of the scyphomedusae, *Cotylorhiza*, and *Nereis*. Vitamin C shows a very particular localization in *Aplysia* (Fig. 63); it is scattered throughout the cytoplasm of the immature egg but accumulates later in the region of granules located in a ring at the periphery of the egg. During cleavage, ascorbic acid passes into definite blastomeres (CD and later 3c and 3d). The interest of these observations is somewhat lessened by the fact that ascorbic acid is lacking or does not undergo any characteristic

changes in the other species studied. One must not forget that Barnett and Fisher have cast doubt on the possibility of detecting ascorbic acid *in situ* and have studied the errors in the method adopted by Ries.

The reaction given by the —SH group in most forms studied

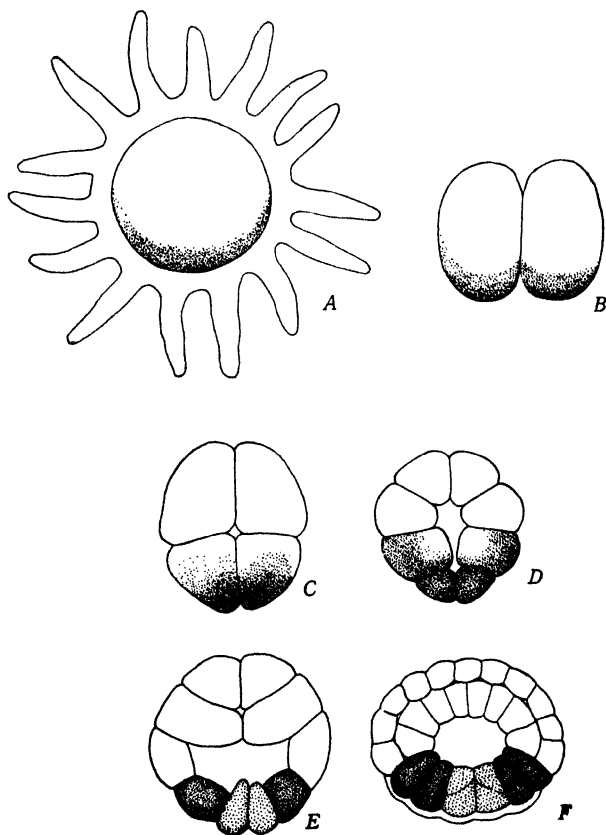


Fig. 64. Localization of phenolases in *Ciona* (Ries).

is especially intense in the region of the germinal vesicle of the oöcyte, for Ries has not been able to detect any other localization of substances containing the sulfhydryl radical. The behavior of peroxidase (Fig. 64) is most interesting in *Aplysia* and *Ciona*. In the first case, the reaction is given by all parts of the immature oöcyte, but one notices during maturation a gradual shift of the colored zone into the vegetal hemisphere which is rich in yolk. Thus only the macro-

meres give the reaction. In *Ciona* the reaction is obtained only in a crescent already present in the unfertilized egg. This crescent is bisected by the first cleavage plane, and its later behavior shows it to be identical with the "yellow crescent" of Conklin, which contains the material destined for the development of muscles (myoplasm). The sea urchin and *Chaetopterus* eggs do not contain peroxidase.

Turning to indophenoloxydase, we see that the fats take on the color of the Nadi reaction only in *Aplysia*. In the egg of the sea urchin, the reaction increases greatly at fertilization as we have seen in Chapter IV. It is given especially by the vitelline membrane and the blastocoel fluid, its intensity decreasing at gastrulation. As in the sea urchin egg, there is no selective localization of indophenoloxydase in the egg of *Chaetopterus*. On the contrary, the distribution of this enzyme parallels that of peroxidase in *Ciona* and is contained exclusively in the region of the myoplasm. Similar results are obtained when the recoloration of the leuco derivative of methylene blue by the egg is studied. Finally, the reducing power toward vital dyes is particularly strong in the vegetative part and the blastomeres derived from it. In the sea urchin egg the cytoplasm shows very strong reducing power under anaerobic conditions and its  $rH_2$  drops to 6; but no localization of the reducing systems of the egg are seen.

Figures 63 and 64 illustrate the two most characteristic examples. In Figure 63 the localization of ascorbic acid in *Aplysia* is shown and in Figure 64 that of the phenolases in *Ciona*.

Ries concludes from these investigations that in the mosaic forms there is a juxtaposition of regions which are chemically distinct. The sea urchin egg, on the contrary, is much more homogenous. Ries thinks it is premature to attribute a morphogenetic role to all the substances which he has described. However, one may conceive that the potentialities of the various regions are controlled by the proportions of the substances present. In any case, morphogenesis can not depend on relatively simple factors such as the bipolar differentiation of Spek or the graded variations in respiratory metabolism (metabolic gradient of Child) or in the distribution of a substance (Boveri). Ries believes that the enzymes he has studied are not bound to mitochondria, but that they are distributed in the cytoplasm, relating this conclusion to the observations of Holter already discussed in Chapter III. Holter, however, has already firmly stated that his conclusions are valid only for those enzymes which he has examined. Recalling that amylase is

bound to the mitochondria in ameba he cautions against generalizing from his own observations. Furthermore, we must not forget that for the sea urchin egg we have good reason to believe that the oxidative enzymes are bound to ultracentrifugable granules (Navez and Harvey, Shapiro, Ballentine: see Chapter III). The observations of Stern and of Chantrenne reported in Chapter VI show that the same situation holds for adult organs. Ries' ideas are hardly in line with current ideas on the question of whether the respiratory enzymes are bound to granules or whether they are dispersed in the hyaloplasm of the egg; but this is really only a matter of detail.

Ries later extended his results to other eggs and other enzymes. With Gersch, he followed the reducing power of certain eggs and their capacity to reoxidize leuco derivatives. It is only in mosaic eggs where clear differences are found between different regions, but these differences are not seen at the same stage, nor do they show the same topography from one species to the next. In 1938, Ries took up again the study of *Aplysia* and examined the effects of centrifugation on the distribution of discernible substances. The essential result was that normal development is only possible when the substances separated by centrifugation recombine again. The table opposite gives an idea of the cytochemical composition of the normal and centrifuged egg.

These findings have been confirmed and extended by Peltrera: he noted that the egg of *Aplysia* is capable of a greater degree of regulation than had been supposed, and, from his point of view, the different plasms of the egg correspond to "cytochemical equilibria" which can not be disturbed without making development abnormal. Moreover, none of the distinguishable constituents have any significance as an "organ forming substance."

The effect of centrifugation on the cytochemical structure of the egg has been studied in *Limnaea* by Raven and Bretschneider. Sedimentation of the egg material into four layers results: a lipid cap, formed especially of glycerides; the hyaloplasm, containing the nucleus or the spindle which is characterized by an intense reaction of the —SH groups; a zone of granules, probably mitochondria, which contains indophenol oxidase, peroxidase and probably vitamin C; and finally the yolk proteins. Centrifugation is followed by a redistribution of these egg substances. First the egg regains its homogenous structure, and later the different substances are relegated to definite regions of the egg which give rise to the various organs.

Constituents of the normal egg	pH	rH <sub>2</sub>	Staining	Recoloration of methylene blue	Peroxidase	Nadi	Vitamin C	Fe	K
<b>Cytoplasm of the animal pole</b>	About 8	Oxidizer	Basophilic	+++	—	—	—	—	(+)
<b>Yolk lipids</b>	—	—	—	—	—	+++	—	—	—
<b>Granules</b>	Acid	—	—	—	—	—	+++	—	—
<b>Yolk proteins</b>	About 6	Reducer	Acidophilic	—	+++	—	—	—	—

Constituents of the centrifuged egg	Recoloration of methylene blue	Peroxidase	Nadi	Vitamin C	Fe	K
<b>Fats</b>	—	—	(+)	—	—	—
<b>Hyaloplasm</b>	++	± to ++	—	—	+	+
<b>Yolk proteins</b>	—	±	—	—	—	—
<b>Granules</b>	—	—	—	+++	—	—



The important question of the relation existing between the myoplasm of the ascidians and their oxidative enzymes has been taken up again by Ries and by Reverberi and Pitotti (1939). In *Ciona*, the yellow crescent destined to be included in the muscles selectively gives the indophenol oxidase and the peroxidase reactions; this situation has also been found in other species (*Ascidella*, *Ascidia*, *Phallusia*). Conklin, who discovered the morphological significance

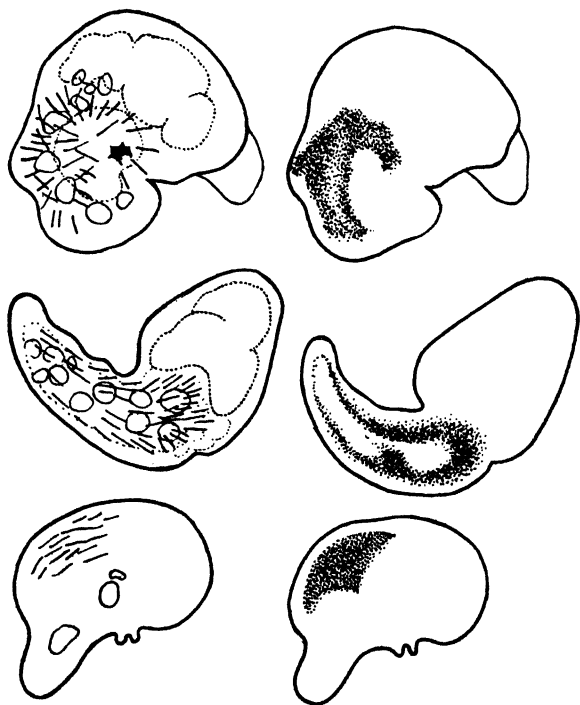


Fig. 65. Localization of muscle (*left*) and peroxidase (*right*) in the ascidian tadpoles which develop from centrifuged eggs (Ries).

of the yellow crescent, rejected the idea that the pigment is necessary for the formation of muscle on the basis of centrifugation experiments. The mitochondria may be eliminated from the myoplasm by centrifugation without modifying its development. According to Ries the situation is quite different with regard to the oxidases. If the blastomeres containing the myoplasm are removed, the remaining blastomeres form tadpoles without muscles and in which the oxidase reactions are lacking. When the internal structure of the egg is

altered by centrifugation, the region which gives the oxidase and peroxidase reactions coincides regularly with the place where muscle differentiates. Figure 65 represents three abnormal ascidian larvae: the peroxidase reaction (at the right) is shown only by the muscle (shown on the left).

Ries concludes that the oxidative enzymes constitute one of those "physical and chemical differences in the hyaline cytoplasm" which Conklin held responsible for morphogenesis in the ascidians. In passing, Ries notes that centrifugation does not bring about a perfect stratification of these eggs, but leads rather to crescents than to regular layers. He attributes this result to a reticular structure of the cytoplasm in accordance with Frey-Wyssling's theory.

The investigations of Reverberi and Pitotti on various ascidian eggs are in full agreement with those of Ries. The Italian authors report that the indophenoloxidase reaction is given by the ventral three quarters of the unfertilized egg, but at fertilization it is restricted to a region in the vegetal hemisphere. At the four blastomere stage, only the two posterior cells which give rise mostly to muscle are colored, and finally, in the gastrula, the reaction is strictly localized in the presumptive muscle cells. Reverberi and Pitotti have further investigated the oxidative enzymes in fragments of unfertilized and fertilized eggs. In the first case, both halves contain indophenoloxidase and each of them can form a normal larva. After meridional fragmenting of the fertilized egg, each of the fragments contains the enzyme and develops normally; but if an equatorial cut is made, the vegetal half alone gives the reaction and it is known that it segments like an entire egg showing bilateral symmetry. The animal fragment, containing no oxidases, is characterized by an abnormal cleavage leading to a radial symmetry. The authors cautiously add that it would be too much to think that the bilateral or radial symmetry results from the presence or absence of oxidases. This appears to them as absurd as the notion that the oxidases present in the presumptive muscle cells are the "final cause" of the muscles. One sees in Figures 66, 67, and 68 how the unfertilized and fertilized eggs behave after sectioning.

In later work (1940), Reverberi and Pitotti again studied the localization of the oxidases in various mosaic eggs. In *Nereis* and *Eucharis*, they could easily follow the distribution of these enzymes from the fertilized egg to the larva. Blastomere D at the four-celled stage is characterized by a large amount of phenolase, and this large

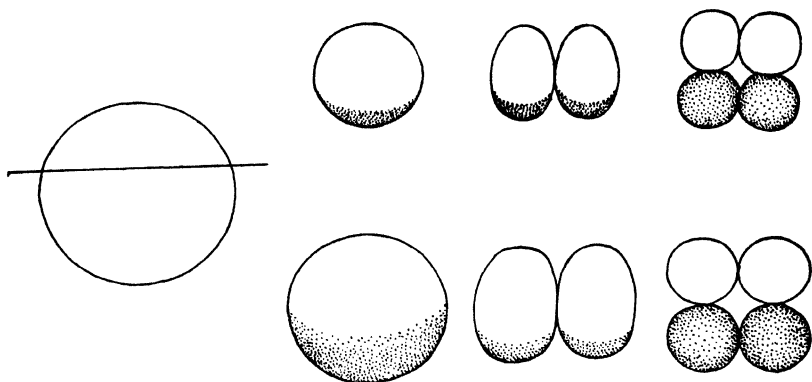


Fig. 66. Effect of a horizontal section of unfertilized ascidian egg upon distribution of the phenolases (Reverberi and Pitotti).

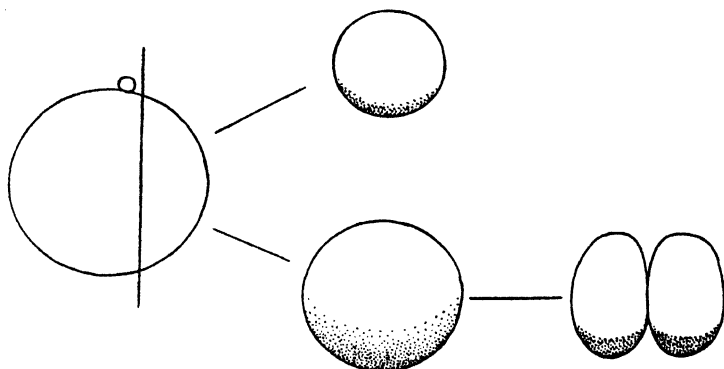


Fig. 67. Results of a meridional section of fertilized ascidian egg upon distribution of the phenolases (Reverberi and Pitotti).

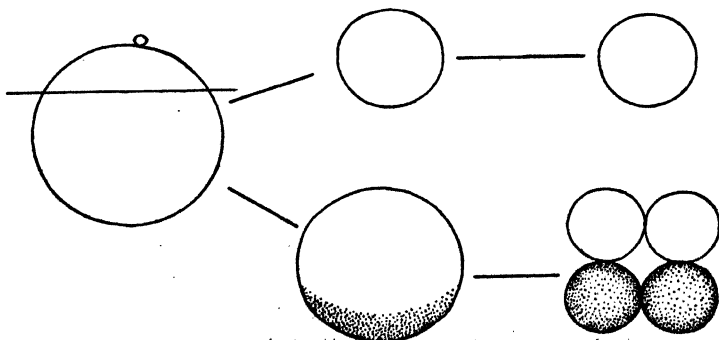


Fig. 68. Results of sectioning fertilized ascidian egg above the equator upon distribution of the phenolases (Reverberi and Pitotti).

blastomere is especially important in morphogenesis, giving rise to the somatoblasts. Subsequently, the peroxidases and the phenolases pass into different blastomeres. In the case of the ctenophore *Eucharis*, the peroxidases exhibit the same localization as that of the fluorescent green pigment in *Beroë* as described by Spek. This pigment, according to Spek, has an organ-forming role. However, the above investigators could not demonstrate any precocious differentiation in the mosaic eggs of *Hydroides*, only the larva showing a localization of the oxidases in this form. This aberrant case is not enough to invalidate the thesis of Ries in the opinion of Reverberi and Pitotti, but they feel, however, that it is desirable—before adopting a definite stand—to extend the studies to other eggs, especially to those showing regulation.

The indophenol oxidase reaction has been used to good advantage by F. E. Lehmann, who worked with the egg of *Tubifex*. Here the color is diffuse before fertilization, but after the extrusion of the second polar body it becomes limited to the "polar plasm" close to the maturation spindle. This region, as has been shown by Novikoff especially, has a unique morphological significance. The indophenol oxidase then passes into the ectodermal and mesodermal somatoblasts which have an important role in the development of *Tubifex* as shown by the work of Penners.

It is obvious that the application of cytochemical methods to the eggs of invertebrates by Ries has brought to light a number of facts of great importance, and it is appropriate now to discuss their exact significance. The experiments of Ries clearly confirm the hypothesis of a more complex structure of mosaic eggs as compared with regulative eggs. There are sufficient intermediate types between the sea urchin egg and those of the ascidians or *Aplysia* so that the results of Ries are acceptable, even by embryologists who repudiate the distinction between mosaic and regulative eggs. Nevertheless, it must be recognized that the methods used by Ries have not demonstrated definite localization of oxidases in certain mosaic eggs. This is true of the polychaetes *Chaetopterus* and *Pomatoceros* and, according to Reverberi and Pitotti, of *Hydroides*; thus, the phenomenon described by Ries, interesting though it is, is not quite general. The techniques that he used will certainly be much further applied, guiding the experimental embryologists in their search for early localizations of certain plasms. The work of Reverberi and Pitotti marks the first step in this direction, for it has con-

tributed to the explanation of the experimental results obtained by Dalcq in the ascidian.

Now let us see what information may be gleaned about the chemistry of invertebrate eggs from the research of Ries. Cytochemical work does not aim merely to give us information about the localization of the different plasms which make up the egg; it also seeks to define the chemical constitution of these plasms. Here we meet with difficulties. We have already seen in Chapter I that cytochemical techniques are subject to severe criticism. Let us briefly consider the more serious of them. Concerning the detection of ascorbic acid, which gives such beautiful results in the ascidian, one is surprised that this very soluble substance should be strictly localized in the granules. Furthermore, the reaction of Giroud, used by Ries, is not completely specific and is also given by melanin, especially. It is unfortunate that Ries has not determined ascorbic acid in extracts of eggs in order to control the results showing its presence in *Aplysia* and its absence in those forms which do not reduce silver nitrate. The fact that the eggs of many species are in this latter group shows that ascorbic acid in itself does not play an important morphogenetic role. All that can be said is that in *Aplysia* there are granules which reduce acid silver nitrate and that they are localized in definite regions during development.

In regard to the phenolases, we know that no unequivocal conclusions can be drawn. The color formed may be instantaneously reduced by dehydrogenases (Keilin), or it may also be that the oxidation of the reagent takes place in a diffuse manner and that the colored product becomes secondarily bound to certain granules as suggested by Hollande. Spek (1938) has made this last criticism of Ries and it is unfortunate that the necessary controls were not carried out. For example, it would be interesting to know if the yellow crescent of ascidian eggs would take on the blue indophenol or the benzidine blue colors when the eggs are placed in solutions containing these substances. This check seems all the more necessary since Lindahl has stained the eggs of the sea urchin with benzidine blue after they had been fixed in a mixture recommended by Pitotti for the detection of peroxidase. The results are superposable on those of the peroxidase reaction, both methods staining the nucleus and the granules of the endoderm cells. It must be concluded, then, that in the case of the peroxidase reaction the nuclei and basophilic granules stain secondarily with benzidine blue. The

same difficulties appear in the case of indophenol oxidase in that the fact that the yellow crescent of ascidian eggs contains certain fats which easily oxidize the Nadi reagent and bind the indophenol blue firmly is enough to obtain the conditions described by Ries and Pitotti and Reverberi. The danger of secondary adsorption of the color is exemplified by a simple experiment by Spek, in which, when he added a precipitate of  $\text{SiO}_2$  to the reagents used by Ries, a blue color developed; it is obvious that the  $\text{SiO}_2$  used does not contain an oxidase.

We must not lose sight of the fact that the results given by the Nadi method for detection of indophenol oxidase are far from being in complete agreement with the biochemical findings involving improved techniques. We have seen in Chapter IV that the intensity of the Nadi reaction increases at fertilization in the sea urchin egg and that Ries concludes that a synthesis of respiratory enzyme occurs. The measurements of Runnström, Örström, Krahle, Keltch and Clowes all agree, however, that the amount of this enzyme in the egg does not change at fertilization, and a more recent paper by Lindahl (1939) has further established that the indophenol oxidase of the unfertilized egg is as active as that of the fertilized egg. He explains the results of Ries by an increasing affinity of the granules of the egg for indophenol blue after fertilization. Brachet has reported (1934) that the frog's egg contains a respiratory pigment, cyanide sensitive and apparently similar to indophenol oxidase, but it does not give the Nadi reaction. In addition, Harrison at the end of a very complete study, concluded that the intensity of the color obtained with paraphenylenediamine is not parallel with the amount of oxygen absorbed when this substance is added.

These few examples are sufficient to show the risks involved in drawing a biochemical conclusion from cytochemical data. In reality it is not demonstrated yet that the yellow crescent is the only region of the egg containing phenolases; moreover, it is not even certain that these enzymes are present. The question will not be solved until the time comes when we can carry out determinations of cytochrome oxidase in the anterior and posterior blastomeres of the four-cell stage. If only the latter contain the enzyme then the information derived from cytochemistry will become valid.

In justice to Ries it must be said that he was very prudent in his conclusions and that he only touched superficially on the role played by the enzymes which he described in the oxidations of the

cell. However, in a more recent paper (1942) on a more theoretical plane, Ries has withdrawn his reservations, and considers that the methods for the detection of the oxidases demonstrate differentiated cytoplasmic regions in mosaic eggs. In his opinion the unsuccessful cases using these techniques are simply a result of the imperfections of the actual procedures. Now, the cytochemical tests used by him would permit the detection of the haemins which play a role in cellular oxidations. This participation is proved, according to Ries, by the increased intensity of the Nadi reaction at fertilization in the sea urchin egg, while it does not change in the egg of *Chaetopterus*. But we know that the respiration of this latter egg decreases to about a half at fertilization and this would imply a less intense Nadi reaction. Ries thus comes to the conclusion that the various regions of the egg are different in the intensity of oxidations, in the  $rH_2$ , and, in the last analysis, in the mosaic of haemins which control respiration. These facts are not encountered in regulative eggs, but, as Ries himself recognizes, quantitative measurements only can supply the proof.

Ries thus claims there is a coupling between the intensity of oxidations and the content in oxidases, from which it would be expected that the posterior blastomeres of the four-cell stage in the ascidian embryo respire more rapidly than the anterior cells. In fact, oxidases are present only in the yellow crescent which is incorporated into the posterior blastomeres. The question deserved an experimental study which was made by Holter and Zeuthen, who studied the respiration of blastomeres isolated from *Ciona* by means of the micro-Cartesian diver of Zeuthen. The results show that the oxygen consumption of the first two blastomeres is the same, as was to be expected, since the plane of cleavage coincides with the plane of bilateral symmetry. But the same respiratory rate was found for the anterior and posterior blastomeres of the four-cell stage and these have entirely different fates. The differences in oxygen consumption between the first four blastomeres of *Ciona* are less than 20%, which is the margin of error admitted by Holter and Zeuthen, and, consequently, only one conclusion is possible. The fact that the yellow crescent selectively shows reactions for oxidases does not mean increased respiration of the blastomeres which contain it; *there is no indication in favor of the idea that the various regions of the mosaic eggs are distinguished by particular respiratory intensities.*

What general conclusions may we adopt under these circum-

stances? Cytochemical techniques constitute convenient procedures for demonstrating the various plasms in mosaic eggs, defining the structure of the egg and allowing us to appreciate its complexity, but they furnish hardly any decisive information on a biochemical level as long as they are not supplemented by other methods. They have hardly any more value than the usual histological methods from the chemical point of view, but they allow us further analysis on a strictly embryological basis. Research of this nature is so important that one hopes it will be extended much further on condition that the experimenter inquires into the causes of error and the dangers inherent in all biochemical interpretations. Above all he must avoid drawing conclusions regarding the rates of respiratory exchange which cannot be measured by the Nadi and the benzidine reactions. Finally, we know now, thanks to Holter and Zeuthen, that regions of different presumptive fates do not necessarily differ in the rate of oxidations.

## 2. Morphogenesis and Metabolism in the Sea Urchin

It was Driesch who first established the fact that the isolated blastomeres of the sea urchin egg were able to regulate almost perfectly, each of them forming a normal pluteus of reduced size. The later research of Boveri, also bearing on separation of blastomeres during development, revealed differences along the animal-vegetal axis of the egg, the blastomeres from the animal half of the morula behaving differently from those coming from the vegetal pole. Boveri explained these results by assuming the presence of an animal-vegetal *gradient*, certain substances necessary for morphogenesis showing a regular decrease in concentration from one pole to the other.

Runnström (1928, 1929, 1931) repeated the classical experiments of Herbst dealing with the action of ions on sea urchin development, and envisaged the egg as the site of two antagonistic gradients. He observed that when one inhibits the development of the vegetal half *animalization* is obtained, that is, an exaggeration of the animal structures, such as the ectoderm and ciliary tuft; conversely other agents produce *vegetalization*, the ectoderm being reduced while the mesoderm and endoderm become excessively large. Vegetalization is a consequence of an inhibition of the animal gradient when the balance between the two opposing gradients is upset and the vegetal



properties predominate. Analogous reasoning explains animalization.

The magnificent experiments of Hörstadius confirm in a striking fashion the hypothesis put forth by Runnström. Here we will merely report the essentials of this work which has been reviewed in all the recent books on experimental embryology; Hörstadius himself has published a complete review (1939). If one isolates the blastomeres of the animal region of the morula, they form blastulae with hypertrophy of the cilia comparable to those resulting from animalization of the whole egg, but when cells taken from the vegetal half are added to these blastomeres they develop more normally, the animalization being the more reduced when the added cells come from more vegetative regions. We must conclude that the vegetative factor, which antagonizes the animalizing factor, decreases in concentration from the vegetal pole toward the animal pole. Con-



Fig. 69. Diagram of a double gradient in sea urchin egg. D, dorsal side; V, ventral side (Dalcq).

versely, if one explants isolated vegetal blastomeres, they give rise to gastrulae with an excessively large digestive tract, and often the endodermal material fails to invaginate during gastrulation resulting in exogastrulae. These anomalies can be corrected by the addition of material of animal origin; here again, it is the cells closest to the animal pole which are most effective. Thus, following the scheme in Figure 69, the egg is considered to contain two antagonistic gradients opposed in direction.

Lindahl deserves the credit of having transposed the problem to a biochemical level by studying the metabolism of animalized and vegetalized larvae. Unfortunately the method of isolation and transplantation of blastomeres, which was so valuable in Hörstadius' experiments, was not applicable because the methods for measuring

metabolism at this time were not sensitive enough. Lindahl hence adopted Herbst's methods and those of Runnström in order to obtain sufficient animalized and vegetalized larvae. He used chiefly lithium chloride to produce embryos predominantly vegetalized, while

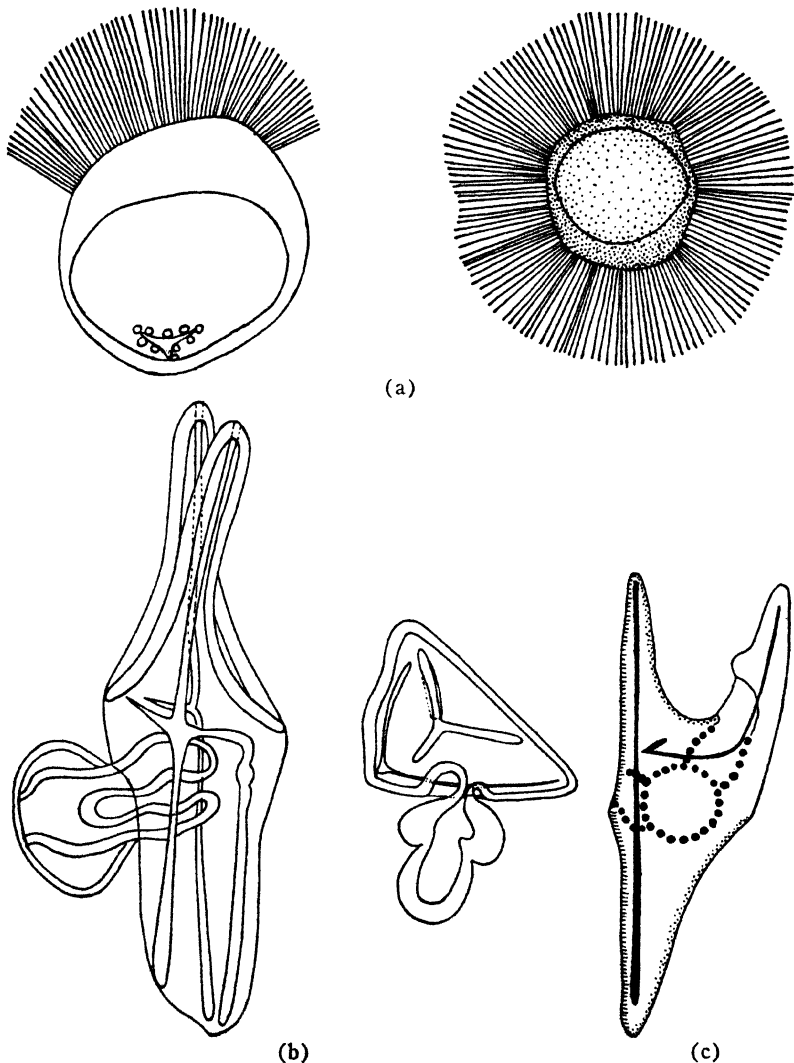


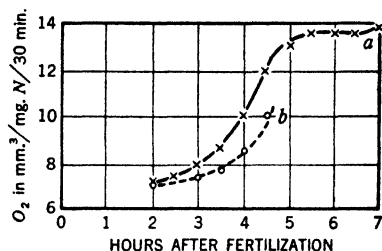
Fig. 70. Two animalized sea urchin larvae (a); two vegetalized larvae (b); and a normal pluteus (c) (Lindahl).

animalization was achieved in various ways; treatment by sulfate-free sea water or by calcium-free sea water followed by sulfocyanide or iodide. In Figure 70 the animalized and vegetalized plutei are compared with the normal.

The techniques utilized by Lindahl to stimulate abnormalities in development present certain difficulties, for, in addition to the specific action of the chemicals on the animal or vegetal gradients, there is a general toxic effect, sometimes exhibited as a simple retardation of development. Lithium, for example, may, according to the conditions, inhibit the animal factor, stimulate the vegetal factor or retard segmentation. Thus it is difficult to determine at what time the specific action on development of these reagents ceases.

First let us examine the action of *lithium*: this ion has its maximum effect when it acts just after fertilization. Earlier it was seen

Fig. 71. Respiration of (a) normal sea urchin eggs and (b) lithium treated eggs (Lindahl).



that the respiration of the sea urchin egg increases during the first 8 hours following an S-shaped curve. Now, Lindahl has shown (1939) that it is at this time that lithium, at an 0.081 *M* concentration, inhibits respiration to the greatest degree, acting chiefly on the rising portion of the curve, as shown in Figure 71. Lithium, then, does not inhibit respiration at the time when it is added, but prevents further increase in oxidations.

Lithium depresses gaseous exchange to a greater degree when it is used in higher concentration: it is likely that a chemical combination between lithium and some constituent of the egg is formed which obeys the law of mass action. What can be the nature of this combination? In 1936, Lindahl showed that lithium poisons certain reactions characteristic of glycolysis. For example, it retards the anaerobic reduction of methylene blue by egg breis in the presence of hexosephosphates, decreases respiration and fermentation of yeasts, and inhibits respiration of egg breis in the presence of hexo-

sediphosphate, coenzyme, and pyocyanine (Lindahl and Öhman). In the last case the reaction obeys the law of mass action. From this assemblage of facts Lindahl formulates the following hypothesis: lithium inhibits the animal factor, and since it also impedes carbohydrate metabolism one may consider that *the animal half of the egg is characterized by the preferential use of sugars*.

Lindahl conceives of the respiration of the egg as being bipartite, composed of two parts: a *constant* portion, which corresponds to the respiration of the fertilized egg, and an *increasing* portion which is sensitive to lithium. Now, if lithium really acts in a specific fashion on carbohydrate metabolism, one may predict that the increase in respiration corresponds to the oxidation of sugars. Some tests have been undertaken to demonstrate the justification of this interpretation by adding to eggs various inhibitors of carbohydrate metabolism, but the results have not been very good. Monoiodoacetamide, notably, causes the respiration to drop to zero, while glyceraldehyde affects chiefly the constant portion of the gaseous exchange. However, these agents produce a rapid cytolysis and their mode of action must certainly be different from that of lithium (Lindahl and Öhman; Lindahl, 1939). The R.Q. measurements carried out by Öhman are more favorable for Lindahl's hypothesis. We have seen earlier that the R.Q. of the egg changes from 0.73 to 0.85 between the second and the eighth hour after fertilization. This latter value would agree with predictions if the R.Q. of the constant portion remained at 0.73 and that of the increasing respiration attained 1. In other words, the egg would utilize fats at a constant rate and would oxidize sugars when the respiration rises. It should be recognized that this argument is still largely a hypothesis and that it can not be considered as firmly established by reason of the complexity of the R.Q. Only precise measurements of the sugars and lipids would lend conviction to the idea, but those which are now at our disposal (Ephrussi and Rapkine, Hayes) were not made at sufficiently close intervals to be really useful. However, they are not opposed to Lindahl's interpretation of the increased R.Q. during segmentation.

Whatever may be the case, certain facts reinforce the idea that the inhibition of the increasing fraction of respiration by lithium is related to its vegetalizing action, this being more marked as the action of lithium on respiration increases. In addition, lithium is especially effective as regards vegetalization at the time when the

increase in respiration is most rapid (between 3 and 5 hours after fertilization), precisely when oxidations are most inhibited.

When lithium is applied at later stages some very different effects are obtained: both respiratory components are depressed, following a reaction which has a temperature coefficient apparently different from that which is obtained for the reaction between lithium and the egg at earlier stages. Lithium, acting in advanced stages, appears to be more a cytolyzing than an animalizing agent; it seems to alter especially the finer structure of the cells. Thus, it is only during the first phase of development that lithium shows specificity, in regard to morphogenesis and cellular oxidations.

Lindahl has made many attempts to combine lithium with various agents which affect metabolism specifically: cyanide, CO, and anaerobic conditions partially depress oxidations and enhance vegetalization. Acting singly, these toxic factors have little effect (Runnström, 1928; Hörstadius and Stromberg). Carbon monoxide alone sometimes causes animalization, sometimes vegetalization; it accentuates animalization of isolated animal blastomeres and vegetalization of explanted vegetal cells (Hörstadius and Stromberg). One might imagine that poisons and anaerobiosis would stimulate the accumulation of acid products; we know in fact that the sea urchin egg forms acid in the absence of oxygen (Runnström). Lindahl (1940) has noted that lactic, formic, and acetic acids appreciably enhance the vegetalizing influence of lithium. Although the base diethylamine possesses the same property it may be that the egg reacts to its penetration by forming acids. It may also be noted that *o*-dinitrocresol, which *increases* respiration, likewise intensifies the action of lithium. Thus there does not appear to be a simple relation between vegetalization and the intensity of metabolism. In face of these difficulties, Lindahl is reduced to the conclusion that all the agents tried have the common effect of making the cytoplasmic structure coarser.

The conclusion that there is no direct relationship between the anomalies of development and metabolism is further confirmed by an investigation of Moore, Bliss, and Anderson who have established that lithium, which lowers oxygen consumption, and pyocyanine, which increases it, are not antagonistic in their effects on morphogenesis. Probably these two substances influence different enzymes in the chain of cellular oxidations.

Let us go on to examine the mechanism of *animalization*. It is interesting to note (Lindahl 1936) that this alteration in development can be effected only under aerobic conditions, treatment of the unfertilized egg with sulfocyanide or iodide ceasing to be effective when the oxygen tension falls below 2% or when cyanide is added. The addition of pyocyanine, which increases respiration, accelerates and intensifies animalization appreciably (Runnström and Thornblom); this abnormality in development might then be linked directly to oxidations, especially those which pyocyanine increases. Unfortunately, it is not possible to measure oxygen consumption at the time when eggs are being treated for animalization, since the shaking of the manometers inhibits animalization perhaps by altering certain labile structures in the egg.

However, measurements are possible after eggs have been treated with animalizing agents. Lindahl has shown that the respiration of eggs is inhibited in increasing amounts by the addition of iodide. According to Horowitz, sulfocyanide prevents gastrulation and leads to permanent blastulae whose oxygen consumption remains constant although that of normal blastulae increases steadily. This observation is found to be in accord with the ideas of Tyler, who, as we have seen earlier, believes that gastrulation requires energy. It is well to add that, according to Horowitz, the inhibition of respiration by sulfocyanide precedes the appearance of visible morphological disturbances.

Animalization is also obtained by placing blastulae in sulfate-free sea water (Herbst). An inhibition of the development of the vegetal half ensues with abnormalities in development slightly different from those obtained by treating unfertilized eggs with sulfocyanide. Lindahl (1936) reported that this abnormal development is accompanied by a lowering of oxidation. He suggests, as an explanation, that the sulfates function in a detoxification of the egg by combining with toxic phenols: he thinks, on hypothetical grounds, that these are waste products of protein catabolism and from this assumes that *protein metabolism predominates in the vegetal half of the egg*. This interpretation receives support from a number of observations. For example, eggs discharge into the medium substances which inhibit development (Peebles), and the amount of these substances increases sharply when the eggs are raised in sulfate-free sea water (Lindahl and Stordal). These toxic agents may be

concentrated by extracting eggs raised in sulfate-free sea water with ether (Lindahl, 1941). It is also noteworthy that the isolated animal half is not affected by lack of sulfates while the explanted vegetal hemisphere undergoes a particularly marked inhibition. One must then conclude that the animal half does not produce these toxic substances, but that they arise at the vegetal pole and diffuse through the membrane. Lindahl and Stordal subjected the egg to various phenol and indol derivatives without very significant results, only K indoxylsulfate causing animalization, and that with quite specific modifications.

The animalizing or vegetalizing influence of various chemical substances on isolated blastomeres has been studied by Hörstadius and Stromberg. The most significant fact that appears from this work is that both sodium pyruvate and glutamine strongly animalize vegetal fragments. The other agents used (extracts of eggs or of animal and vegetal fragments, CO, anaerobiosis, cysteine) do not produce characteristic effects. The authors attempt to clarify the relations between oxidation and morphogenesis in the sea urchin as follows. Pyocyanine and pyruvate, which increase metabolism, favor or produce animalization; cyanide, which depresses oxidations, is weakly vegetalizing, but partial anaerobiosis, which also decreases respiration, has hardly any influence on morphogenesis. Carbon monoxide, which should behave like cyanide, animalizes animal fragments, vegetalizes vegetal halves and has a variable effect on the whole egg. We see that the results are too disparate to postulate a simple relationship between animalization or vegetalization and the intensity of metabolism. Moreover, this conclusion is further justified in view of the fact that, while Tamini has obtained animalization with various agents which stimulate oxidations (such as methylene blue, pyocyanine, *p*-nitrophenol), Lindahl, on the other hand, reports an enhancement of vegetalization with lithium by adding *o*-dinitrocresol, a substance which increases the metabolism of the sea urchin egg considerably.

Leaving for the moment the work of Hörstadius and Stromberg let us consider the point of view adopted by Child, the embryologist who has contributed most to directing attention to the gradient idea, earlier put forth by Boveri. Child considers that morphogenesis is controlled by a *physiological gradient*, whether it be in embryonic development, in regeneration, or in budding. This gradient decreases

progressively along the axis of the organism, being at the highest point on the "axial" gradient where morphogenesis is most active. The physiological gradient of Child is also a *metabolic* gradient, in the sense that the rate of oxidation decreases regularly along the length of the principal axis. He detected physiological gradients by extremely varied procedures. For example, they can be demonstrated by *differential susceptibility* by placing the organism in a toxic substance, where disintegration through cytolysis will begin at the highest point of the gradient. It is the head of a worm which cytolyzes first when treated with cyanide. This differential susceptibility is shown, always in the same manner, by the use of a wide variety of chemical compounds, which do not necessarily influence oxidation in any specific way. The existence of axial gradients may also be demonstrated by following the decoloration of an organism in anaerobic conditions which has been vitally stained with methylene blue or Janus green. Decoloration begins at the highest point on the gradient where metabolism is most active. Child and his students have used some cytochemical techniques, particularly the indophenol reaction and the —SH reaction, and, finally, they have carried out direct measurements of gaseous exchanges, to which we will return later.

Child quickly applied his concept of the physiological gradient to the sea urchin egg, and the ideas of Runnström, Hörstadius, and Lindahl on the subject of a "double gradient" merely represent a refinement of Child's theory. Nevertheless, the latter maintains that the introduction of a second gradient is superfluous and that development of the sea urchin egg is explained if one involves a quantitative variation of a single factor along the animal-vegetal axis, without the necessity of assuming specific regional differences. His explanation is based chiefly on experiments (1936, 1940, 1944) in which he followed the anaerobic decoloration of methylene blue and Janus green in sea urchin and starfish eggs. During oögenesis, the region nearest the nucleus has a more marked reducing power than the remainder of the eggs, and at maturation the reduction begins at the animal pole and spreads toward the opposite extremity. This reduction gradient persists as such up to the end of cleavage, but in the advanced blastula the cells situated at the vegetal pole show an increased reducing power when they begin to invaginate. These cells are derived from the micromeres to which Hörstadius as-



signs considerable morphological importance and which are destined to form the primary mesenchyme. Thus, during gastrulation or a little before it, an activation of the vegetative material occurs, expressed as an increase in its ability to reduce. In the blastula stage, a decreasing ventro-dorsal gradient, coexisting with the primary animal-vegetal gradient makes its appearance. Later the region which forms the mouth becomes in turn a region of especially high reducing activity. The diagrams (Fig. 72) taken from Child summarize

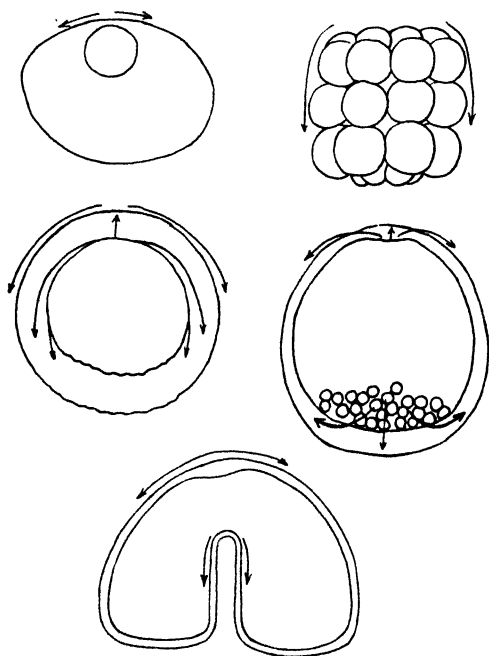


Fig. 72. Reduction gradients during development of *Echinoderma* (Child).

these observations: the arrows indicate the direction of the reduction of the vital dye.

Child concludes that the animal-vegetal gradient is clearly revealed by his method, and that if an opposing gradient exists it has nothing to do with oxidation. Not until the time of gastrulation does the vegetal pole show any particular activity. According to Child there is no reason to believe that in the sea urchin egg there are any qualitative or specific regional differences. He thus rejects

Lindahl's hypothesis of qualitatively different metabolisms at the two poles. He further draws attention to the fact that lithium is not the only vegetalizing agent known, Janus green or insufficient volume of sea water leading to the same abnormalities.

Child's conclusions are not conceded by Lindahl (1942), who particularly insists on the specificity of vegetalization. This abnormality, he says, is not ordinary exogastrulation and, furthermore, if other agents exert effects similar to lithium then they must act upon the same type of metabolism. Since these remarks deal chiefly with the morphological aspects of Child's observations, how are we to consider the biochemical aspects of the reducing power of various regions of the egg?

Child's experiments were repeated by Gersch and Ries who obtained divergent results. The German authors could not see a clear gradient before the advanced blastula stage, and at this time the vegetal cells, which begin to invaginate, are characterized by a *less intense* reducing power than other cells. Gersch and Ries are thus in complete opposition to Child on all points.

For this reason it was especially interesting when Ranzi and his collaborators studied the problem from 1937 on. Ranzi and Falkenheim compared lithium-treated eggs and controls for reducing ability, indophenol oxidase activity, peroxidases, and —SH groups. Like Child they found an animal-vegetal gradient, though weak, the reduction of dyes being somewhat slower at the vegetal pole. They do not comment on the cells originating from the micromeres. Lithium tends to retard reduction at the animal pole and this effect goes hand in hand with the intensity of vegetalization. Indophenol oxidase, peroxidases, and —SH groups are not distributed in gradient fashion, and these constituents of the egg tend to disappear during normal development, although this phenomenon is greatly retarded in eggs treated with lithium. Ranzi and Falkenheim concluded that the  $rH_2$  of the vegetal pole, which gives rise to endoderm, is greater than that of the animal pole, which provides the ectoderm. The low  $rH_2$  characterizing the animal half would correspond to a very high metabolic activity of the protoplasm. A substance inhibiting metabolism would diffuse from the vegetal pole through the membrane, but its influence would naturally be greatest at the vegetal pole itself. Lithium modifies the protoplasmic colloids and by lowering respiration simulates an exaggeration of the vegetal gradient.

Following this, Ranzi studied the action of sulfocyanide on the egg. This substance increases the reducing power of the vegetative material and its  $rH_2$  is thus lowered; animalization would be the result of this change in  $rH_2$  in the presumptive endodermal material. The diagram in Figure 73 illustrates Ranzi's concept.

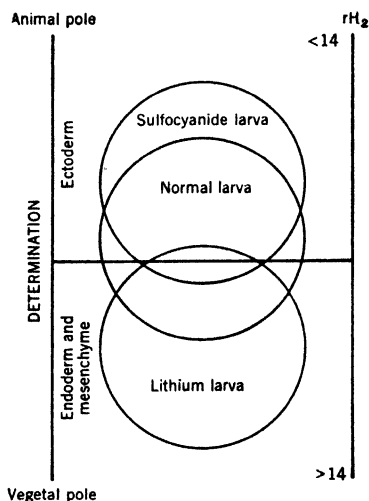


Fig. 73. Determination in sea urchin egg and  $rH_2$  (Ranzi).

Summarizing, the Italian author believes that the determination of the egg depends chiefly on its  $rH_2$ . A region with great reducing power and thus a low  $rH_2$  tends to form ectoderm. If this region increases in size, a part of the vegetal material will be animalized; if, on the contrary, under the influence of lithium, the zone of low  $rH_2$  is reduced, a vegetalization is obtained. Ranzi prefers the following scheme to that of the double gradient: *the egg forms a uniform field in which the micromeres secrete a substance inhibiting metabolism, and this results in an increase in the  $rH_2$  of the vegetal pole.*

One of Ranzi's collaborators, Pitotti, has studied peroxidases in the sea urchin egg after fixation by a mixture of alcohol and chloroform. The reaction is positive up to the blastula stage and then becomes much weaker in the animal half. Lithium reduces this almost negative zone, while sulfocyanide increases it. But Lindahl

(1940) showed that the method used entails grave errors. The difficulties arise from the fact that benzidine blue chiefly stains the nuclei, which are more numerous in the vegetal half; Pitotti's technique does not permit a distinction between the ecto- and endoderm on a chemical level—as she believed.

At this point we find ourselves faced with three hypotheses for the explanation of morphogenesis in the sea urchin egg. (1) Lindahl holds that at each pole there is a qualitatively different metabolism, the intensity of which gradually falls off in passing to the opposite pole. At the animal pole carbohydrates are utilized, while protein catabolism characterizes the vegetal pole. (2) Child conceives of a single reduction gradient which decreases from animal to vegetal pole; a second center of activity appears at the vegetative pole about the time of gastrulation. (3) Finally, according to Ranzi, the egg is homogenous but a substance produced at the vegetal pole by the micromeres lowers metabolism and increases  $rH_2$ ; in this way an inequality of the reducing power of the two poles is attained.

We may now briefly discuss these three ideas. It is clear that Lindahl alone has used methods which are free from criticism and thus has a solid foundation for his experiments. Nevertheless his hypothesis still rests on weak evidence. Although he has clearly demonstrated that *in vitro* lithium decreases the rate of oxidation of sugars by egg breis, he has not yet proved that this ion affects carbohydrate metabolism in the living egg. Furthermore, we do not know whether lithium may also depress other reactions, since Lindahl's experiments were limited to the oxidation of hexosephosphates. Also, we must recognize that Lindahl's attempts to use specific inhibitors of carbohydrate metabolism have not furnished the results anticipated. J. and D. Needham have recently re-examined this question and find that *dl*-glyceraldehyde, monoiodoacetate, and phlorizin fail to give vegetalization. This leads them to reject Lindahl's hypothesis. If it is agreed that lithium exerts an action on morphogenesis different from the effects of these substances, then the nature of the chemical reactions which it affects remains unknown. It is also certain that substances other than lithium are vegetalizing, and there is no reason to believe that they inhibit carbohydrate metabolism. The arguments in favor of a protein metabolism at the vegetal pole are still more inconsistent, assuming as they do the existence of a phenolsulfatase with no known localization; one is not even sure

that it serves to bind the phenols originating from protein breakdown. In addition, Lindahl remains very vague on the question of whether or not lithium retards development of the egg. If it does, one would explain the fact that it inhibits the increasing fraction of respiration in quite a different way, since this increase is parallel to the amount of cell division. It could be that the depression of oxidation is simply the result of the slowing of development and of morphological abnormalities. The question is all the more important since MacArthur definitely observed a retardation in development by treatment with lithium. Be that as it may, the concept of Lindahl has the advantage of being in harmony with the results of Hörstadius. It has unquestioned interest, but at present is still a hypothesis. Clearly, it is rash to believe, as do certain embryologists, that lithium specifically inhibits carbohydrate metabolism while sulfocyanide impedes that of the proteins; such an assumption is certainly possible, but it remains to be proved.

The ideas of Child and Ranzi suffer from the inadequacy of the techniques used, especially since they are not quantitative. It must be pointed out that Gersch and Ries failed to find the reduction gradient in the egg. The explanation suggested by Child tends to fix the activity of the micromeres at a late stage (advanced blastula), but the investigations of Hörstadius show that these cells contain the vegetalizing factor from the beginning of segmentation. In this regard his explanation seems less plausible than that of Lindahl. Ranzi's theory is especially hypothetical. There is nothing to show, for example, that a low  $rH_2$  is evidence of a high metabolism; the  $rH_2$  of an egg is a complex average of the different oxidation-reductions taking place. If one system predominates in one hemisphere of the egg, its  $rH_2$  will be different from that of the other half without necessarily changing the rate of oxidation. There is no evidence that the higher  $rH_2$  of the vegetal pole is caused by the liberation of an inhibiting substance; the redox systems of the two halves of the egg may in fact differ qualitatively. Here again it is a matter of plausible assumptions that lack experimental proofs.

It is clear that the results of these investigations remain somewhat deceptive. Lindahl's careful and competent research suffers from the fact that it was necessary to work with large numbers of eggs because of the low sensitivity of the methods. For this reason he was not able to attack directly the question of the metabolism

of the isolated animal and vegetal halves and was limited to an analysis of the anomalies caused by chemical agents such as lithium and sulfocyanide, the specificity of which agents is not perfect. Lindahl was well aware of these deficiencies, and since the perfection of the Cartesian diver technique he has attacked the problem anew with the collaboration of Holter. The comparative measurements of the oxygen consumption of animal and vegetal blastomeres undertaken by Lindahl and Holter were carried out on 10 to 20 eggs in each experiment, the authors taking care to define the possible sources of errors of the method by controls with other procedures and by making sure that the preliminary removal of the fertilization membrane of the egg did not change its metabolism. They verified the equality of the respiration of the sum of the animal and vegetal fragments with that of the whole egg. These preliminary experiments indicated that the method has a precision of 2 to 3%; to be on the safe side, the authors consider that differences of less than 10% are within the limit of experimental error.

The chief point of interest in the work of Lindahl and Holter is the comparison between the oxygen consumption of isolated animal and vegetal halves. Their careful and numerous measurements show conclusively that there is *hardly any difference between the two types of blastomeres*. In fact, the oxygen consumption of the vegetal half is  $98.7 \pm 1.7\%$  of that of the animal half (average of 15 experiments). The increase in respiration during cleavage is shown by both halves of the egg and to the same degree. The authors then compared the respiration of the animal and vegetal fragments after allowing them to develop. Here again the strongly animalized and vegetalized larvae absorb the same amount of oxygen, and, in addition, the sum of their respective oxygen consumptions is equal to that of the whole embryo at the same stage.

Lindahl and Holter continued their studies by examining the effect of various poisons on the animal and vegetal blastomeres. Glyceraldehyde depresses the respiration of the animal portion by 27%, that of the vegetal regions 32%; the respiration of the entire egg is reduced by 40%. Cyanide inhibits the oxidation of animal and vegetal fragments to about the same extent (73 and 71%, respectively). A similar result is found for lithium, a concentration of 0.154 *M* decreasing oxidation 29% for the animal halves, 30% for vegetal fragments, and 32% for the entire egg. Finally,

pyocyanine stimulates the respiration of the two types of blastomeres to about the same degree (+ 21% for the animal half, + 18% for the vegetal cells, and + 22% for the entire egg).

These interesting results prove clearly that *the sea urchin egg does not possess an animal-vegetal respiratory gradient*. The reality of such a gradient was not categorically claimed by Child and Ranzi, but their results led them to consider it very probable. The fact that lithium inhibits the respiration of both types of fragments to the same extent demonstrates that the "increasing" fraction of the respiration is not limited to the animal pole. Furthermore, we have just seen that the metabolism of the two halves of the egg increases by the same amount. Likewise, pyocyanine, which affects only the "constant" of respiration, exerts the same action on both fragments, so it must be concluded that the two fractions of the respiration are distributed homogenously along the animal-vegetal axis. The same situation holds for the cyanide-sensitive portion of respiration.

If the precise work of Lindahl and Holter did not give the expected solution, it has, nevertheless, brought to light some valuable facts. We may conclude that their investigations furnish no argument in favor of a respiratory gradient in the sea urchin egg, and that the existence of different metabolisms at the two poles is likewise improbable. Whether oxidation at the two poles of the egg is concerned with distinct substances is not yet clear: only measurements of the R.Q. of the two halves would give a decisive answer. We await with curiosity and interest the studies of Lindahl and Holter on this problem.

We may add here the results of investigations by Lindahl and Holter dealing with the localization of dipeptidase in the egg. This enzyme to all appearances plays a very important role in protein metabolism. Holter, Lanz, and Linderstrøm-Lang, using a dilatometric method, compared the amount of dipeptidase in animal and vegetal blastomeres at the eight-cell stage and observed no difference. Later, Lindahl and Holter extended this conclusion to the entire developmental period up to the pluteus stage. At no time did they find an accumulation of this enzyme in either the animal or vegetal half. However, to be exact, one can not exclude differences in *activity* of peptidase in various regions of the egg, since the method used only measures the quantity of the enzyme.

There is a last point to consider. We have been concerned thus

far with the possible differences between the animal and vegetal poles of the egg, but what is known about the determination of the *dorso-ventral* axis? If one makes a meridional section of the two- or eight-cell stage (Driesch, Hörstadius), the plutei which develop possess a dorso-ventral organization. Thus determination in this direction is still labile at these stages and regulation is almost perfect. Lindahl (1932) reported that if the unfertilized egg is drawn out by passing it through a narrow capillary and then fertilized, the anterior end always becomes ventral. This artificial orientation of the dorso-ventral axis is reversed if the anterior end is stained heavily with Nile blue sulfate. Lindahl thinks that the anterior end, by reason of the stretching in the capillary, has an increased respiration, and that Nile blue sulfate, being toxic, would lower the respiration locally. It may be cited in favor of Lindahl's idea that stretching of eggs by ultracentrifugation increases their oxygen consumption (Velick), the activity of their indophenol oxidase (Navez and Harvey) and of their dehydrogenases (Ballentine). However, Lindahl (1941) reported that in some experiments carried out with Holter he did not find any increase in oxidations caused by stretching of the egg. These eggs were forced through capillaries and thus one must conclude that the increase in metabolism obtained by Velick is related to the rearrangement of the egg contents rather than to elongation.

In this regard it is interesting that centrifugation will also determine the dorso-ventral axis (Runnström, Lindahl, Pease), the ventral side appearing in the region containing the cell inclusions. A very careful study by Pease indicates that a factor which determines the ventral side of the noncentrifuged egg also enters into play. Thus Pease was led to assume that this side is determined by the collaboration of two factors, a dorso-ventral gradient anchored in the cortex and not changed by centrifugation and a substrate scattered in the cytoplasm which the centrifuge sediments. The ventral side will appear at the place where these two factors, cortical and central, are found in highest concentration.

These ideas relate to some recent observations by Monné, who compared the structure of centrifuged and normal eggs with the aid of the polarizing microscope. This author concludes that the fibrils making up the "structural phase" (see page 184) are oriented parallel to the cortex, and show slight differences between the two poles. Centrifugation does not change the structure of the cortex, but it displaces



the structural phase in the interior of the egg, and it is the accumulation of this phase in a definite region which stimulates the formation of the ventral half. The ribonucleoproteins in this phase would play an essential role in this phenomenon by stimulating growth and the synthesis of proteins. In normal development, too, the ribonucleoproteins would accumulate in the more active morphogenetic regions: at the micromeres and ventral side in the sea urchin, the dorsal half of the egg in amphibians.

Finally, Pease has attempted to clarify the role of respiratory metabolism in dorso-ventral determination in the sea urchin. Pease placed embryos at the 8-cell stage in a concentration gradient of various substances influencing metabolism; this was carried out by a simple and ingenious method: the eggs were placed on a porous glass filter covered with sea water for 2 hours; the other side of the filter contains the solution to be studied. The filter is impregnated with Nile blue sulfate so that the lower pole of the egg which is exposed to the higher concentration is stained as well. At the pluteus stage it is easy to determine the relation between the ventral side and the stained area. Pease (1940) found that the ventral side formed at the region of highest concentration of cyanide, 2,6-dinitrophenol, urethan, monoiodacetate, iodine, and ferricyanide. On the contrary, fluoride, arsenate,  $\text{Cu}^{++}$  ions, pyrophosphate, and malonate are ineffective. Later he observed that sodium azide ( $\text{NaN}_3$ ), pilocarpine, pyocyanine, cysteine, and carbon monoxide also exert a positive effect, the lower surface of the egg giving rise to the ventral side with a higher frequency. Paraphenylenediamine and lithium chloride had no action on dorso-ventral determination. Pease concluded that there was no correlation between inhibition of mitosis produced by some of the substances tried and the appearance of the ventral side, the latter being determined not by any sort of growth gradient but by a specific enzyme system. The fact that KCN, CO, and  $\text{NaN}_3$  are active suggests the participation of cytochrome oxidase. The role of dinitrophenol and pyocyanine would be explained by their activity on the dehydrogenases, and that of cysteine may be in relation to reversible changes in the proteins. It appears that the phenomenon is very complex from the chemical viewpoint, and it must be pointed out that some of the substances which have opposite actions on cellular metabolism exert the same effect on the egg.

### 3. Metabolism of Insect Eggs

The study of embryonic development of insects has made great progress thanks to the studies of Seidel and his school (see Krause). In the egg of a libellulid, Seidel has described the presence of an early *formative center* localized in the posterior part of the egg, this center functioning by producing a chemical substance which diffuses forward. Later, a second center, located more anteriorly, begins to function. This *differentiation center* does not act by secreting a substance but by contracting the yolk mass. The intimate mechanism of the contraction of the yolk, indispensable for morphogenesis, certainly deserves physicochemical study. One might assume, with J. Needham, that the fibrous molecules in the yolk shorten at this time. The chemical embryology of the insect egg at these early stages remains unknown because of the technical difficulties of the operations on this material, but measurements carried out on single eggs are now possible and it is hoped that investigators will no longer be discouraged. The only data with regard to the formative center indicate that this region is often identified with a region staining deeply with thionine. Some research, as yet unpublished, by Ten Cate and Mulnard (personal communications) carried out on the eggs of various species of insects, has established that this basophilia is due to the presence of ribonucleic acid. The cytoplasm near the periphery of the egg and to which the nuclei migrate at the end of segmentation is also rich in ribonucleoproteins.

*Drosophila*, which is such favorable material in genetics, now appears to be equally good for chemical embryology. Recent work by Boell and Poulson represents a meeting place of biochemistry, embryology, and genetics. Important work by Poulson shows that the early stages in development are profoundly affected by deficiencies in the X chromosome. Its complete absence results in an abnormal migration of the nuclei to the anterior end of the egg, which dies at the end of segmentation. Absence of a part of the X chromosome, which is responsible for the normal migration of the nuclei, produces the same anomaly; if, on the other hand, the other part of the X chromosome is lacking, segmentation is normal but gastrulation shows such irregularities that the embryo soon dies. Boell and Poulson have measured, by means of the Cartesian diver, the oxygen consumption of eggs lacking the X chromosome (YY eggs). Respiration is identical with that of the controls and in-

creases in normal fashion until development is arrested; then it begins to decrease, so much so that it falls to one-fifth of the metabolism of the control and then remains constant for about fifteen hours. If one determines the oxygen consumption of eggs having a supplementary Y (XXY) or a supernumerary X (XXX), perfectly normal values are found. Thus it must be concluded that respiration is modified only when development is arrested and that the chromosomal formula does not induce any changes in gaseous exchange when development is normal. This result agrees with Tyler's ideas concerning the energy demands during morphogenesis. The fact that the genetic constitution has no repercussion on respiration also agrees with observations on centrifuged eggs and on enucleated oöcytes (see Chapter III) on the minimal role of the nucleus in metabolism.

The biochemical analysis of mutations producing anomalies at later stages in development has begun in the United States and has already shown promising results. Thus, Poulson and Boell have studied a mutant which, in the embryo, has a nervous system three times the normal size although abnormal. Its cholinesterase activity is about three times higher than normal, indicating that the nervous system of the mutant has about the same cholinesterase activity per unit volume as the control. In addition, Villee has ascertained that the oxygen consumption of the imaginal disks of the wings is reduced in comparison with the normal in mutants in which the wings are smaller in the adult (vestigial or minute); but that there is no difference in the respiration of the imaginal disks of the legs of the mutant and wild type.

We owe to Bodine and his collaborators a valuable study of the metabolism of the orthopteran egg in advanced stages of development. It is chiefly an analysis of metabolism during *diapause*, a latent period during which growth and development are completely arrested. Gaseous exchange at this time is simply that of maintenance, agreeing with Tyler's theory. Therefore, we would expect to find respiration lower at diapause than at the stages preceding and following it. This is indeed what Bodine found (1929) in a grasshopper, *Melanoplus differentialis*. The oxygen consumption of eggs in diapause drops to a level 4 to 6 times lower than that of the embryos in prediapause and ascends when development begins again (Fig. 74).

Bodine later examined the effect of cyanide (1934). This

poison inhibits the respiration of developing eggs intensely, although it has no action on the embryos in diapause, these eggs behaving like the unfertilized sea urchin egg while the embryo before and after diapause is comparable with the fertilized sea urchin egg. The eggs behave as if a respiratory enzyme, sensitive to KCN, does not function during diapause. A careful re-examination of this question by Robbie, Boell, and Bodine has not changed the main conclusions. They found that the respiration of freshly fertilized eggs is mildly sensitive to KCN, although at this time the oxidations are as yet very low. As metabolism increases during prediapause the effects

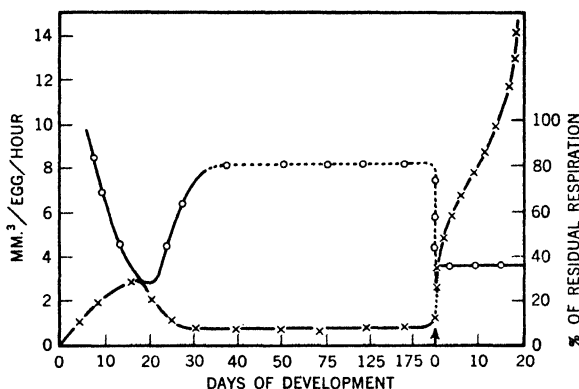


Fig. 74. Oxygen consumption of normal eggs (x, ordinate on left); inhibition of respiration by KCN (O, ordinate on right) in *Melanoplus*. Diapause is between 20 and 175 hours (Robbie, Boell, and Bodine).

of cyanide become more marked, but are reduced, on the other hand, when oxidations decrease at diapause. After this latter stage, the gaseous exchanges increase and become KCN-sensitive. Figure 74 indicates that cyanide is only effective during stages of intense metabolism.

Carbon monoxide inhibits respiration of eggs in pre- and post-diapause stages but intensifies it during diapause. Here again there is a perfect parallel with the observations of Runnström on the sea urchin egg (see Chapter IV). These facts have also been found by Wolsky in the silkworm. This author accepts the explanation proposed by Runnström and considers that the respiratory enzyme is not saturated by its substrates during diapause.

The oxygen consumption of grasshopper eggs does not depend on their iron content, which remains constant throughout development (Bodine and Wolkin). It may be that the iron combined with cytochrome oxidase undergoes some changes, but Bodine and Boell (1936) have found that the amount of indophenol oxidase does not decrease when diapause ensues. However, this enzyme is synthesized during post diapause, according to Allen. The American authors rightly insist that there is no relation between the amount of indophenol oxidase present in the egg and the amount of KCN-sensitive respiration, there being no direct relationship between the physiological function of the enzyme and its ability to oxidize paraphenylenediamine. These observations are yet another warning against hasty conclusions one may be tempted to draw from cytochemical studies on indophenol oxidase. In addition, Bodine and Boell point out that ultracentrifugation of the egg does not modify its metabolism during diapause while this treatment lowers it before and after the resting period. This decrease involves chiefly the fraction sensitive to KCN; hence, centrifugation reduces the percentage of physiologically active respiratory enzyme without, however, changing the amount of the enzyme in the egg. This result can easily be explained since we know that centrifugation sediments the granules containing the respiratory enzyme, these granules and the enzyme thus losing their normal relations with the other components of the chain of oxidations (Chantrenne). In effect, ultracentrifugation produces a "desaturation" of the respiratory enzyme in the sense which Runnström considered. Nevertheless, other interpretations may be invoked to account for the low respiration of the egg in diapause. Perhaps there is a disappearance at this time of one of the intermediate hydrogen "carriers," a decrease in the activity of the dehydrogenases, or a blocking of the respiratory enzyme through the formation of a labile complex in the cell.

Bodine and Boell (1937, 1938) have also followed the effects of substances stimulating oxidation. Methylene blue stimulates a considerable increase in the respiration of the egg in diapause, this increased metabolism being then sensitive to carbon monoxide. On the contrary, methylene blue has no effect on embryos of pre- and post-diapause where the inherent respiration is high. Nevertheless, dinitrophenol increases oxidation in all stages; this intensified respiration is cyanide-sensitive and is characterized by an abnormally high

R.Q. and an increased oxidation of proteins resulting in increased ammonia excretion. It may be noted that the grasshopper egg proves to be very favorable material for demonstrating Needham's theory of the succession of sources for metabolism (see preceding chapter), as the measurements of R.Q. (Boell) and carbohydrate breakdown (Hill) show. Bodine has also found that there is a direct parallel between oxygen consumption and uric acid production during all of development.

This brief treatment shows that the orthopteran egg presents a series of attractive problems from both the point of view of the mechanism of cellular oxidations and that of the energetics of development; this egg, like that of the sea urchin, constitutes favorable material for the study of factors which stimulate metabolism at a time when development is stimulated. Its study furnishes many facts conducive to Tyler's ideas about the necessity of a utilizable source of energy for growth and differentiation.

#### 4. Conclusions

We may be brief, since it is well recognized that it is hardly possible to grasp with any clearness the play of chemical factors which operate in morphogenesis in the invertebrates. The reasons for this must be sought in the inherent difficulties of the material itself. It has been only a short time since techniques which permit a frontal attack on the question have been available, but the work accomplished to date has been very profitable. It has prepared an indispensable foundation for research to come and, without a doubt, we will soon be in a position to strike much closer to the heart of the problem.

The cytochemical observations on regulative and mosaic eggs have an undisputed value, for, in combination with the methods of experimental embryology, they bridge many gaps. Unfortunately, they do not permit us to go very far in a biochemical direction. We must not lose sight of the fact that if the ascidian egg behaves quite differently from that of the sea urchin from the point of view of cytochemical reactions, it is not the same for oxidation, for, in both cases, the various blastomeres have the same oxygen consumption whether or not they have different fates. The task of the future will consist chiefly in determining the real nature of the heterogeneities which are revealed by cytochemical techniques. Progress can not fail to

be rapid as soon as the main clues are discovered. It does not appear that oxidative enzymes form the really essential elements in development. If this were true, one could not explain the identical respiration of the anterior and posterior blastomeres in *Ciona*. It is more probable that the regions demonstrated by the phenolase reactions contain granules or specific proteins which may have a very fundamental role.

Concerning the sea urchin egg it may be said that its development is not governed by a quantitative gradient in metabolism, the difference in reducing activity between the two poles of the egg, from point of view of oxidations, playing only an accessory role. This does not exclude the possibility that this factor participates in morphogenesis, but at present no proven fact upholds the possibility. As yet, one can not give an opinion regarding Lindahl's hypothesis of the presence of qualitatively different metabolisms at the two poles of the cell. However, this explanation has the merit of being open to experimental verification and the results of these tests will not be long in appearing. The pursuit of research in this field is very necessary. Since Hörstadius' experiments can be comprehended only if the notion of differences at the two poles is accepted, all efforts tending to define their physicochemical nature will be awaited with interest.

## CHAPTER IX

# Chemical Embryology of Amphibian Eggs

The chemical transformations undergone by the eggs of vertebrates during their development have been the object of numerous investigations, analyses having been carried out chiefly on the chick embryo and the amphibian egg. The latter is the more favorable for a study of the initial stages of development, the chick embryo scarcely being accessible for chemical studies before the second or third day of incubation, by which time early morphogenesis is ended the very period which attracts our attention here. Although the hen's egg forms good material for the biochemist, it does not offer the same advantages for a study of the chemical basis of morphogenesis. The amphibian egg lends itself both to experimental and biochemical observations from fertilization on, and this double advantage has resulted in much research on this material during recent years. The fact that the center of interest has shifted from the chick embryo to the amphibian egg is particularly revealing of the new tendencies in chemical embryology today. Now the problem of morphogenesis is at the fore, while the chemical reactions which insure the transformation of the yolk into the developing chick embryo are relegated to second place. Thus we refer the reader who is interested in the chemical embryology of the hen's egg to Needham's book (1931). The problems which are considered there have made little progress since Needham discussed them. However, it is appropriate to discuss here some of the work of Needham and his collaborators on carbohydrate catabolism; we will return to it shortly. As to the meager data on the metabolism of the chick embryo in early stages, they may be intergrated into this chapter without much difficulty,



where we will deal exclusively with the metabolism of the whole egg and postpone until later the question of differences in metabolism in different regions of a single embryo.

### 1. Toxic Substances and Development

Useful information on the metabolism of the egg can be obtained from studies on reactions to poisons which specifically affect certain chemical processes. We must also take into account substances that stimulate particularly curious anomalies in embryogenesis.

We owe to Bellamy, one of Child's students (1919, 1922), the first systematic study of the effect of various poisons on the frog's egg. The idea behind his experiments was to elucidate, if possible, "differential susceptibilities" in certain regions of the egg. It will be remembered that the selective sensitivity of definite portions of an organism to poisons is considered by Child as an indication that they are the regions of highest metabolic activity. In the frog's egg, Child (1928) claimed the existence of a primary gradient, decreasing from the animal to the vegetal pole, a second gradient, with a second center of high metabolism, appearing at gastrulation. This special region would be the dorsal lip of the blastopore and would thus correspond to the organizer of Spemann. The dorsal lip of the blastopore would thus constitute a "dominant" region capable of controlling the development of certain regions which are subordinate to it. If Child's idea is correct, we would expect to find a particularly high physiological activity first at the animal pole and later in the region of the blastoporal lip, such activity being revealed by a more marked susceptibility to poisons. This is just what Bellamy found in following the effects of formaldehyde, potassium permanganate, sublimate, magnesium chloride, sodium butyrate, ethyl alcohol, and potassium cyanide on development. The effect of these substances on metabolism is very diverse; cyanide combines with heavy metals and thus blocks respiration; formaldehyde reacts with the  $\text{NH}_2$  groups of the proteins, while sublimate blocks the  $-\text{SH}$  groups; permanganate may oxidize the latter, while alcohol and  $\text{MgCl}_2$  are anaesthetics. Bellamy's main conclusion is that the animal pole of the morula or the blastula is characterized by a clear differential susceptibility, by rapid necrosis or the blocking of segmentation in this region. At gastrulation it is the dorsal lip of the blastopore which becomes most sensitive to the toxic agents (Fig. 75).

Bellamy's work calls for a few comments. First it is regrettable that he restricted himself to a superficial examination of the embryos without making a histological control and that he did not check the effects of the substances used on the metabolism of the egg. It is also to be noted that the action on the gastrula was sometimes limited to disturbances during invagination, without selective cytolysis of the blastoporal lip. Now, these anomalies are very common, often being found in batches of eggs that are not perfectly mature. One therefore cannot consider them as a specific disturbance of the organizer, since we know now that there are means of dissociating invagination from induction to a large degree, as shown by the experiments on ultraviolet radiation by Dürken and those on centrifuging by Pasteels. In their cases, closure of the blastopore was obtained without the formation of a nervous system. This anomaly, which

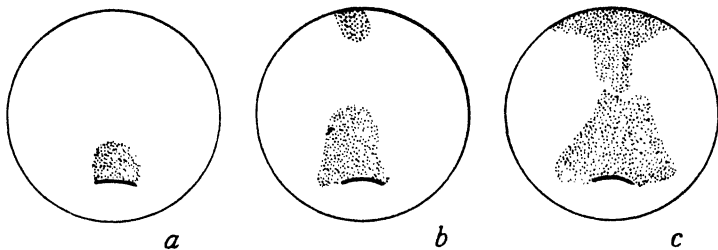


Fig. 75. Three stages in cytolysis of gastrula of the frog by chemical agents showing the differential susceptibility of the dorsal lip of the blastopore and of the animal pole (Bellamy).

implies a direct action on the organizer, has not been reported by Bellamy. Furthermore, his experiments show that quite different substances have similar effects, indicating that regions of high differential susceptibility do not necessarily have a more intense respiratory metabolism than others. This method only demonstrates some vague physiological properties, not very accessible to further analysis.

Holtfreter (1943) has shown that the differential susceptibility of the organizer to KCN has nothing to do with its specific effect as a poison for oxidation. Actually it is the alkalinity of the cyanide that accounts for its action and it can be replaced by dilute solutions of KOH. He also showed that the rate of disintegration of different regions depends on the thickness and solidity of the surface coat of the cells and not on a metabolic gradient. This coat is better de-

veloped in active morphogenetic regions, but the cells there are subjected to a greater tangential tension, the result being that a rupture occurs first where the surface coat is dissolved by the alkaline medium. The greater local contraction in these regions results in the exposure of a greater number of cells to the toxic agent and disintegration is more rapid.

Bellamy's results have also been criticized by Cannon, but the doubts raised by the latter have been settled by a convincing answer by Bellamy and Child. We may take it then, that this work shows that two regions, the animal pole in the blastula and the blastoporal lip in the gastrula, are particularly sensitive to toxic agents to which they respond by cytolysis or a blocking of development. However, one can not infer anything regarding the metabolism of these regions.

Let us go on to investigations on the inhibition of *respiratory metabolism* and look first at the effects of anaerobiosis. We already know that it does not prevent segmentation in the frog's egg (Samassa, Godlewski, Bataillon, Parnas and Krasinska, Lennérstrand, J. Brachet). The capacity of more advanced stages to develop in the absence of oxygen, however, decreases continuously: blastulae are prevented from gastrulating, and middle gastrulae are not able to complete gastrulation. Finally, young neurulae are stopped almost as soon as deprived of air, while blockage is immediate for advanced neurulae. If one compares neurulae of different species with regard to their ability to close the neural plate under anaerobiosis, one finds wide differences (Spirito, Barnes, Spiegelman and Moog). Neurulation is complete in *Discoglossus* and the toad while it is immediately inhibited in the frog, *Hyla*, axolotl and lamprey. In the last case, even segmentation is not possible in absence of oxygen. These facts, to which we will return later, show that the presence of free oxygen is not a decisive factor in morphogenesis.

*Potassium cyanide* gives almost identical results (Bataillon, Bellamy, J. Brachet (1934), Spirito). Here again susceptibility to the toxic agent increases during development although considerable variations are found between different species. Recall the interesting fact observed by Spiegelman and Moog and Hall that sodium azide, which generally has an effect similar to cyanide, blocks development from segmentation on. This poison acts on some phosphorylation reactions indispensable to the normal course of mitosis, such as the dephosphorylation of ATP.

This brings us to the inhibitors of *glycolysis*. We have reported (1934, 1939) that moniodoacetate,  $M/1000$ , has no effect on aerobic development of the frog egg, although it stops development at neurulation in *Discoglossus*. This poison appears to accelerate cytolysis when it is used in absence of air. When high concentrations are used ( $M/30$  to  $M/60$ ) segmentation and gastrulation are normal in the frog but neurulation is inhibited, so that one can easily dissociate gastrulation from the formation of the nervous system by means of moniodoacetate. Fluoride  $M/100$  has no influence on development in *Rana fusca*.

The question has been re-examined on an American species, *Rana pipiens*, by Pomerat and Haringa who attempted to find out whether or not frog's eggs exhibit a "nonphosphorylating glycolysis." We know that in muscle and in yeast, fermentation of the carbohydrates goes through a series of phosphorylated intermediates, glycogen first being transformed into a hexosemonophosphate (Cori ester) by means of a phosphorylase. Muscle glycolysis is possible only in the presence of certain phosphorylated compounds, such as adenosine triphosphate and creatine phosphate. According to Needham and his collaborators, matters are quite different in the chick embryo. This form utilizes glucose, not glycogen, and is able to attack it without forming phosphorylated intermediates. Thus the chick embryo would differ basically from muscle in this respect, being unable to transform glycogen or hexosephosphates into lactic acid, containing neither coenzyme nor triosephosphate dehydrogenase. On the contrary, it easily attacks glucose and mannose. This "glucolysis" of the chick embryo may be blocked with dilute glyceraldehyde and with relatively high concentrations of fluoride. However, it must be pointed out that the point of view of the English biochemists is not unanimously shared. Meyerhof and Perdigon have criticized their concepts and have shown that they can demonstrate coenzyme and triosephosphate dehydrogenase in the chick embryo if certain precautions are taken.

To come back to the world of Pomerat and Haringa, they reported that development in *Rana pipiens* is quickly stopped by large doses of fluoride ( $M/40$ ), while it is not affected by weak concentrations ( $M/200$ ). This egg is very sensitive to moniodoacetate, which blocks segmentation even in concentrations of  $M/1500$ . Iodoacetamide is still more active because of better penetration. As to

glyceraldehyde, it is ineffective perhaps because it is destroyed by the living egg. From their investigations the American authors conclude that glycolysis without phosphorylation occurs in the amphibian egg, but their argument is weak since the action of these substances on metabolism was not studied. The fact that fluoride is effective in only large doses could be due to insufficient permeability of this substance. Lacking any measurements, one cannot accept as demonstrated that the egg carries out glucolysis and not glycolysis. Finally, there is nothing to show that the explanation of the lack of effect of glyceraldehyde is correct. Furthermore, Barnes, also working on *Rana pipiens*, did not obtain a clear effect with monoiodoacetate until the end of gastrulation, thus confirming earlier results on *Rana fusca*.

We have had occasion to study, in collaboration with L. Rapkine, the effects of iodoacetamide on the eggs of *Rana fusca* (unpublished observations) and to compare them with another toxic agent which reacts readily with —SH groups, chloropicrin (Bacq). Both substances stop development in low concentrations, iodoacetamide thus being much more active than the corresponding acid, a fact already observed by Pomerat and Haringa. The ineffectiveness of monoiodoacetate is probably due to poor penetration into the egg because of the dissociation of the molecule. Iodoacetamide and chloropicrin block development at all stages. During segmentation, they stimulate a rapid degeneration of the spindle and the asters, possibly as a result of combination with the —SH groups present in the proteins making up the achromatic figure (see Chapter V). An interesting anomaly, that we will consider later, appears in gastrulae treated with these poisons and then returned to water. Embryos form with very defective nervous systems but with the adjacent notochord and somites normally formed.

Ten Cate noted that blastulae or gastrulae of axolotl treated with arsenate often show microcephaly and he assumed that the arsenate blocks the oxidative decarboxylation of sugars and the Pasteur-Meyerhof reaction. It is possible, however, that here again the action consists in the blocking of the —SH groups of proteins or of enzymes.

Let us say a word concerning the action of *narcotics*. We know that they decrease the activity of dehydrogenases (Keilin), but they also affect the physical properties of cytoplasm, particularly its vis-

cosity (Heilbrunn). Thus their influence is hardly specific. The agents mainly used have been alcohol (Bellamy, Heller, Tamini), ethylurethan, chloral hydrate (Parnas and Krasinska), trichlorobutyl alcohol (Marx), phenylurethan (J. Brachet). The effects decrease during development, the reverse of that occurring with cyanide. Segmentation is soon stopped, but at the end of neurulation disturbances are no longer observed. The chief alteration appears to consist in an inhibition of the gelation of the cytoplasm during mitosis which results in a blocking of cytoplasmic division while nuclear division continues. At the same time oxidations are reduced some 30 to 50 per cent.

It should be noted that certain substances accelerate embryonic development in the amphibians probably by increasing the level of oxidation. This is the case of dinitrophenol (Andreassi), pyocyanine (Citterio), Nile blue (personal communication of Dr. Fautrez); thionin, on the other hand, is without effect (Chiunini). Dawson found, however, only a retardation with dinitrophenol, even in low concentrations, this substance blocking development in blastula and gastrula stages.

What may we conclude from all these experiments? It is evident that specific inhibitors of metabolism do not affect morphogenesis in any selective manner. One sees no direct relation between oxidations and glycolysis, for example, and gastrulation and neurulation. Sensitivity to cyanide and anaerobiosis increases during development, it is true, but it shows variation from one species to the other. Glyceraldehyde is without effect, while fluoride acts only in high concentrations. Iodoacetamide and chloropicrin produce rapid blocking which leads one to suspect that —SH groups are indispensable at all stages of development. We will see shortly the results of the few investigations where care was taken to control the supposed effects of these inhibitors by direct measurements of metabolism.

There are some substances, on the contrary, which specifically affect certain processes of embryogenesis. Unfortunately, in these cases it is their mode of action on the chemistry of the eggs that is unknown. In sea urchin special interest is attached to *lithium* and *sulfocyanide*. Lehmann (1934, 1937) devoted some important investigations to the effects of LiCl on amphibian eggs. The most curious anomalies involve the structure of the head, which shows a

series of malformations: cyclopia (already reported by Leplat), monorhiny, etc. By working at the beginning of gastrulation, Lehmann obtained embryos lacking a notochord but with a rather normal appearing nervous system. The somites extended along the median line where they fused and replaced the notochord (Fig. 76); the

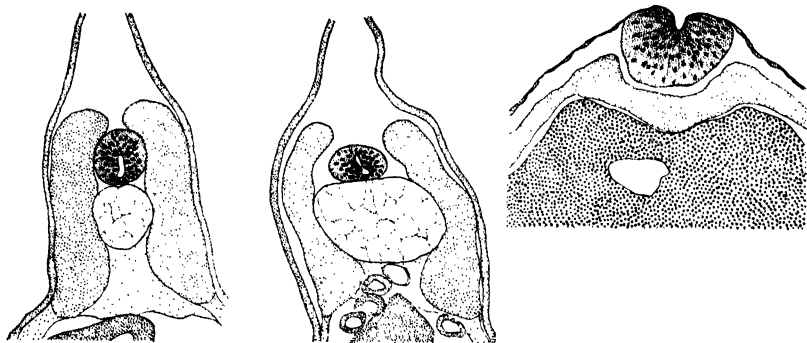


Fig. 76. *At left*, a normal embryo; *center*, embryo of the same stage treated with the NaSCN (Ranzi)—note the increase in the volume of the notochord; *right*, mesodermalization of the notochord by LiCl in a much younger embryo (F. E. Lehmann).

latter thus underwent a “mesodermalization.” According to Lehmann, the anterior (cephalic) region and the caudal region of the nervous system develop to different extents following treatment with lithium in the gastrula stage, this situation revealing differences between the chemical bases for differentiation of the cephalic and caudal nervous system. However, these conclusions are not agreed to by Pasteels, who re-examined this question recently. Embryos without notochords were also obtained by Ranzi and Janeselli by treating lamprey eggs with lithium. They also detected a greater sensitivity of the anterior half or the posterior half of the nervous system according to the stage of gastrulation.

Ranzi and Tamini had the good idea of trying the action of sulfocyanide on amphibian eggs. As in the sea urchin, it has an action opposite to that of lithium, embryos being obtained with an excessively well developed notochord both in length and diameter. The nervous system also shows hypertrophy. Very often the notochord of treated embryos does not elongate normally, but becomes zigzag in shape, with the neurula elongating in an abnormal fashion

(Ranzi, personal communication by J. Pasteels). Figure 76 shows, side by side, an embryo treated with lithium (at the right), a control (on the left), and an embryo treated with sulfocyanide (center). In the chick, sulfocyanide stimulates chiefly anomalies of the closure of the neural folds and retardation of growth, according to Nowinski and Pandra.

Some students of Ranzi have studied the effect of other substances that produce animalization in the sea urchin on the amphibians. Thus Citterio found pyocyanine to act like sulfocyanide in increasing the size of the notochord. The same effect is obtained with *p*-nitrophenol (Bartolazzi), but in a lesser degree: this toxic agent produces chiefly a generalized inhibition of development, with a slower utilization of the yolk and retarded closure of the nervous system. Ranzi (1942) concluded that there is a parallel between the effectiveness of these substances in the sea urchin and in the amphibians, animalization having for its counterpart the development of the notochord.

Hypotheses on the mode of chemical action of lithium and sulfocyanide are, naturally enough, not lacking. In 1937, Lehmann called attention to the analogy between vegetalization in the sea urchin and mesodermalization of the notochord. He suggested, following Lindahl's hypothesis, that lithium depresses carbohydrate metabolism of the gastrula in the amphibians. Ranzi and Tamini (1939) even thought that lithium would inhibit carbohydrate metabolism of the notochord while sulfocyanide would paralyze the breakdown of proteins in the nervous system. A more careful examination of their embryos treated with sulfocyanide shows, however, that the nervous system, far from being reduced, undergoes hypertrophy. The explanations proposed by Lehmann and by Ranzi and Tamini are not very plausible. We have seen that Lindahl's hypothesis is far from demonstrated in the sea urchin, so there is nothing to justify its extension to amphibians, especially since no quantitative biochemical studies have been done. Such studies of the effect of lithium and sulfocyanide on the metabolism of amphibian eggs appear highly desirable.

In the absence of precise information, we are reduced to mere deduction; hence a recent work of Tamini is the more welcome. She treated frog and axolotl eggs with various substances, chiefly the specific inhibitors of carbohydrate metabolism. Her work is,



in short, comparable to that undertaken by J. and D. Needham in the sea urchin. It will be recalled that these authors obtained no typical vegetalization with these poisons. Tamini concentrated on anomalies of the head, cyclopia, in particular, verifying that fusion of the eyes is frequent after treatment by lithium. This anomaly becomes rarer as one substitutes for LiCl the following compounds:  $\text{Na}_2\text{SO}_4$ , NaCl,  $\text{MgCl}_2$ , sodium tartrate, phlorizin, alcohol, and NaF. Monoiodoacetate, glyceraldehyde, and cyanide are ineffective in this respect in the frog. The results of these experiments show that inhibitors of glycolysis rarely induce cyclopia. Tamini hence concluded that the primary action of lithium could hardly consist in a paralysis of carbohydrate breakdown. It seemed more probable to her that lithium owed its effectiveness to its ability to flocculate colloids. This hypothesis is the more attractive because of the growing importance that we tend to attribute to the proteins during development, and it finds a certain experimental support from recent investigations by Ranzi and his collaborators on the effects of sulfocyanide and lithium on the viscosity of extracts of gastrulae. These extracts were prepared by means of concentrated salt solutions (NaCl, KCl) and Lawrence, Needham, Shen and Miall have established that one obtains in this way preparations which show changes in viscosity because of the presence of proteins with elongate molecules. The viscosity of these solutions is increased, according to Ranzi, by the addition of lithium and decreased by sulfocyanide. However, it must be added that some research by Rebuffat and Brachet has shown that the physical properties of these extracts are due in large part to the presence of thymonucleohistone. In addition, lithium slightly increases the viscosity of thymonucleohistone extracted from nucleated red cells, while sulfocyanide decreases it strongly. Since there is no indication in favor of the idea that lithium and sulfocyanide act on the nucleus, it is still doubtful whether the results obtained by Ranzi and his students really furnish the clue to the mystery.

One curious fact that remains unexplained has been reported by Töndury in *Triton* and confirmed by Orts Llorca in the chick. Testosterone and other steroids produce, in low concentration, a unilateral hypoplasia of the nervous system, inducing numerous mitotic abnormalities with greater frequency on the side where development is the more changed.

Finally, it is interesting to report that a number of substances prevent the formation of the lens in spite of a normally formed optic cup. This occurs notably with chloretone (Lehmann, 1934), dinitrophenol (Dawson), urea, NaBr, NaCl, (Jenkinson, 1906), phlorizin (Tamini). The variety of agents renders improbable a specific effect on a special metabolism.

A precise interpretation of these many observations is indeed difficult. Probably amphibian eggs react to various toxic agents by the formation of the same abnormality, hence all deductions on a biochemical level become impossible. Many of the substances combine a general toxicity with a more specific effect. The same difficulties are encountered here as in the sea urchin where it is not always easy to distinguish between vegetalization and ordinary exogastrulation. Lithium is probably no more specific in the amphibians than in the sea urchin. For example, embryos without notochords have been obtained by placing frog eggs at high temperatures (Hoadley), and we have seen that numerous substances induce cyclopia. It may be added that auxin in relatively concentrated solutions, or modifications of the  $O_2$  and  $CO_2$  tension of the eggs (Copenhaver and Detwiler), will produce cyclopia. Some physiological factors, the biochemical significance of which completely escapes us as yet, also stimulate a great variety of abnormalities, such as overripeness of eggs. It is clear that an analysis with biochemical methods is indispensable in order to link together these aberrations of development and to furnish a coherent explanation of them.

## 2. Metabolism of the Frog Egg

### *a. Oxidation and Glycolysis*

It is superfluous to repeat the details concerning the rate of oxygen consumption since this question has already been discussed earlier (Chapters V and VII). It will be recalled that the respiratory quotient has an exceptionally low value during segmentation; Bialaszewicz and Bledowski obtained a figure of 0.60 while Brachet found 0.65. But this value increases sharply at the beginning of gastrulation, when the respiratory quotient of the gastrula approaches unity and is maintained between 0.95 and 1.0 up to hatching, as Brachet reported in 1934 (Fig. 77). Spirito, using another method, observed an R.Q. value of 0.95 during neurulation in the

frog and the toad. Although the interpretation of respiratory quotient values is always dangerous, it seems clear that the morula is the site of incomplete oxidation, and that at the end of gastrulation the catabolism is principally carbohydrate. In any case it is certain in *Rana fusca*, at least, that *the nature of oxidations is modified sharply during gastrulation*. It would be very useful to know whether this is a constant feature in the anurans or a peculiarity of this species since, as we shall see, there is a tendency to attribute very real importance to this qualitative change in respiratory metabolism.

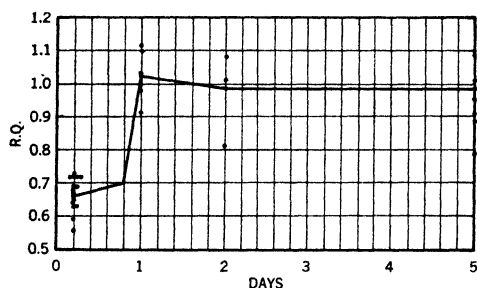


Fig. 77. Variations in respiratory quotient during development in the frog (J. Brachet).

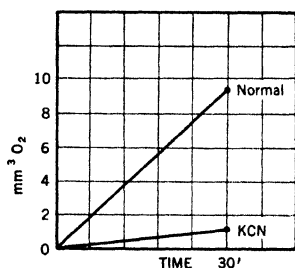
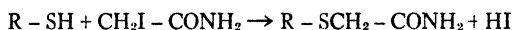


Fig. 78. The action of *M/1000 KCN* on oxygen consumption of frog egg (J. Brachet).

In *Rana fusca* oxidations are strongly inhibited by cyanide which depresses respiration 90 per cent, whatever the stage treated (Fig. 78). The result is that eggs segmenting in cyanide have a respiration of only 10 per cent of the normal. It can be concluded from this extreme sensitivity to cyanide that respiration is catalyzed in the main by an enzyme analogous to cytochrome oxidase. Almost identical results have been found by Barnes and by Spiegelman and Moog on *Rana pipiens* as well as by Philips on the teleost, *Fundulus*.

How do the inhibitors of carbohydrate metabolism affect respiration? Brachet showed in 1939 that monoiodoacetate (*M/30*) did not lower respiration until the time when development was retarded, that is, the end of gastrulation. Inhibition then sets in rapidly and in a few hours attains a value of 90 per cent; the same holds true for *Rana pipiens* (Barnes). Iodoacetamide, which blocks development almost instantaneously, inhibits oxidation much more

rapidly, a concentration of  $M/500$  causing respiration to fall to 25–40 per cent of the normal in two or three hours. This inhibition is found at all stages of development with the exception of unfertilized eggs, which can be treated without injury with iodoacetamide with no change in the rate of oxidation (unpublished observations made with L. Rapkine). Probably this toxic agent does not penetrate before fertilization. We have been able to show that there is a production of hydriodic acid when amphibian larvae are placed in iodoacetamide; this fact leads one to think that the poison acts on oxidation and development by combining with  $-SH$  groups according to the reaction:



Fluoride, as we have seen, does not affect development in *Rana fusca*, nor does it influence the rate of oxygen consumption, although it lowers the respiratory quotient from 0.98 to 0.82 and inhibits lactic acid production about 90 per cent. This decrease in R.Q. and the inhibition of glycolysis are also found in eggs treated with monoiodoacetate (J. Brachet, 1939). However, it must be pointed out that the low rate of gaseous exchange and of lactic acid production make these measurements very precarious, and small errors may profoundly affect the results. We cannot thus say with certainty that fluoride and monoiodoacetate really penetrate the egg and block carbohydrate metabolism without affecting its morphogenesis. However, it appears probable that such is the case.

We shall see now if the study of *carbohydrate catabolism* confirms the interpretation given for the respiratory quotient. If the explanation is correct, we should expect to find glycogenolysis beginning at gastrulation. The work of Fauré-Fremiet and Dragoiu and of J. Needham (1927) has shown that the egg loses about 40 per cent of its glycogen between fertilization and hatching. According to Needham, however, the amount of reducing sugar in the egg, determined after hydrolysis of the entire egg, does not decrease significantly, the glycogen presumably being transformed into another carbohydrate without furnishing any energy for growth or differentiation through its combustion or fermentation. Later investigations by J. Brachet and J. Needham have determined the way in which the egg utilizes its glycogen. Analyses were carried out from day to day on both the free glycogen (lyoglycogen) and the fraction bound to proteins (desmoglycogen). Figure 79 gives the results ob-

tained. It is seen that desmoglycogen forms only a small fraction of total glycogen. It is interesting that it is this desmoglycogen, linked with proteins, which is first attacked. In addition, it will be noted that glycogenolysis begins at gastrulation and continues up to hatching. The measurements of the glycogen content of the egg thus confirm completely the deduction drawn from a study of the respiratory quotient, namely, that the utilization of carbohydrates is not appreciable up to the time of gastrulation, which appears to be a turning point in the metabolism of the egg.

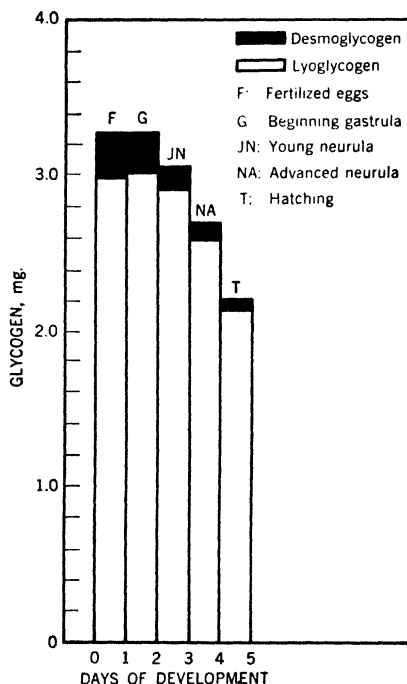


Fig. 79. Utilization of lyo- and desmoglycogen from fertilization to hatching in the frog (J. Brachet and J. Needham).

Somewhat different results have been obtained by Gregg and Pomerat using *Rana pipiens*. The amount of glycogen of the embryo decreases by 23.5 per cent between fertilization and the morula stage, and then increases slowly to attain its original value at neurula-

tion. From this time on, glycogenolysis is resumed and continues beyond hatching.

A few experiments have been done on *lactic acid production* by the frog's egg at different stages. Lennerstrand reported that the amount of this acid in the fertilized egg was very low, in fact, only 7 to 10  $\gamma$  per egg. Later the eggs produce lactic acid, the concentration being doubled at gastrulation and more than tripled at neurulation. Lack of oxygen for seven to eight hours raises the lactic acid content of the morula some 15 to 20 per cent, but the increase under anaerobic conditions becomes much more marked at gastrulation (80 to 100 per cent). When the eggs develop in absence of air for twenty to twenty-two hours, glycolysis greatly increases at cleavage, but this is undoubtedly an injury phenomenon, since Lennerstrand himself reported that some eggs cytolize under these conditions. In any case their development had been stopped for several hours.

Brachet (1934) was able to confirm in a general way the findings of Lennerstrand. The lactic acid content increases during development, the rise being particularly clear at gastrulation. An exposure of fifteen hours to an atmosphere free of oxygen increases glycolysis at all stages. Further, Trifonova, Korovina and Sliussarev have also observed an increase of lactic acid during segmentation. As we have already seen (Fig. 62), this acid tends to disappear at the beginning of gastrulation, its content increasing during the latter process and dropping again at neurulation. Since the Russian authors emphasize the difficulties of analysis of such yolk-laden material, some slight doubt persists regarding the validity of their conclusions.

In addition, according to Barnes, the egg contains a glycogenase which varies in concentration during development. This enzyme, which transforms glycogen into a reducing sugar, is found in appreciable quantities in the unfertilized egg. One finds only about half the amount during cleavage, but it becomes more abundant in the gastrula, where its concentration is 45 per cent more than that of the unfertilized egg. After a brief drop at the end of gastrulation, the enzyme is synthesized continuously during neurulation.

All these findings amply confirm the idea that the morula uses only insignificant amounts of sugars, it being only at gastrulation that the breakdown of carbohydrates becomes really important.

Further variations during gastrulation and neurulation do not appear to be established in a convincing manner.

The intimate biochemical mechanism of carbohydrate breakdown remains poorly known. Nowinski tried to show that the advanced tadpole, free of yolk, carries out glycolysis without phosphorylation, similar to the mechanism suggested for the chick embryo by J. Needham and his collaborators. Nowinski reported that a brei of tadpoles forms lactic acid in appreciable amounts. This glycolysis is increased by addition of glucose while it is not influenced by glycogen or hexosediphosphate. Finally, lactic acid production is 90% inhibited by glyceraldehyde. The presence of a glycogenase (Barnes) speaks in favor of a nonphosphorylating glycolysis.

However, one may present facts opposing this hypothesis. Brachet, in collaboration with Rapkine (unpublished), has found that young embryos contain triosephosphate dehydrogenase—which plays an important role in muscle glycolysis—thus explaining why Nowinski failed to detect an accumulation of triosephosphates when he added hexosediphosphate to a brei of tadpoles. Furthermore, Lindahl and Lennerstrand have definitely demonstrated the presence of cozymase in amphibian eggs. It is noteworthy that the amount of this important coenzyme in the egg increases during segmentation and attains its peak at gastrulation, confirming once again the presence of a direct coupling between this phase of development and carbohydrate metabolism. However, it is clear that certain factors indispensable for carbohydrate metabolism are already present during cleavage, probably in an inactive state. Frog embryos lose much of the coenzyme after gastrulation, containing only traces at the time when muscular contraction sets in.

The question of whether the frog's egg uses glycogen through typical glycolysis or without the participation of phosphates cannot be answered at the present time. Actually, there is no reason why both reactions may not go on together.

In relation to carbohydrate metabolism it is worth while considering the interesting changes in the *acid soluble phosphate compounds*. From fertilization to the end of neurulation a hydrolysis of phosphocreatine is found (Zielinski), resulting naturally in an increase in inorganic phosphate. Later, the amount of phosphocreatine clearly increases; this increase coincides with the appearance of definite muscular movements. Thus it is probable that this

phosphagen plays the same role in embryonic muscle as in the adult.

Development is accompanied by an increase in acid-soluble organic phosphate (Zielinski, J. Brachet, 1939). Unfortunately, the nature of the compound synthesized remains unknown, but it seems that adenosine triphosphate or any other nucleotide can be excluded. After hatching of the embryo, Zielinski (Fig. 80) observed a considerable synthesis of all the acid-soluble phosphates, which probably results from an increased utilization of the yolk reserves. Thanks to the research of Fauré-Fremiet and Dragoiu, we actually know that

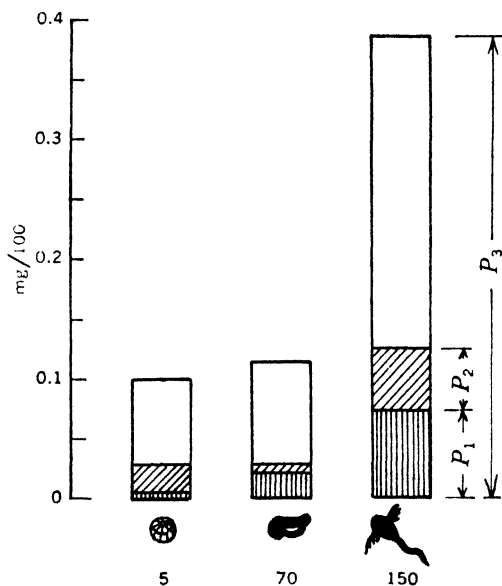


Fig. 80. Metabolism of phosphate compounds during development in the frog.  $P_1$  = inorganic P;  $P_2$  = phosphocreatine;  $P_3$  = total acid soluble P (Zielinski).

the yolk of the frog's egg is rich in phosphorus. Zielinski assumes that the phosphagen serves, in early stages, to keep the amount of adenosine triphosphate in the egg constant when the egg is subject to short periods of anaerobiosis, thus forming an energy reserve ready to be used when the amount of adenosine triphosphate begins to fall. It would be interesting to verify this hypothesis by determination of the phosphate compounds in the egg under anaerobic conditions. A first step in this direction has been made recently by Barth



and Jaeger who have established the fact that frog's eggs in the gastrula stage contain adenosine triphosphate or a substance very similar from the chemical point of view. This compound becomes hydrolyzed under anaerobic conditions with the liberation of inorganic phosphate, and is resynthesized when oxygen is readmitted. Thus the frog egg behaves like muscle and spermatozoa in this regard.

Let us say a few words concerning lipid metabolism, a subject which is still not very well known. Parnas and Krasinska could not detect a utilization of fatty acids during development. The amount of lipid P decreases, however, perhaps because of a utilization of phosphatides for the synthesis of nucleic acid, conforming to Loeb's hypothesis. Bialaszewicz and Mincovna have confirmed the fact that the fatty acids are not consumed until after hatching. However, Fauré-Fremiet and Dragoiu did find fatty acid utilization (about 27 per cent) before hatching, the phosphatides and the unsaturated fatty acids being the only fractions attacked. The disagreement between these findings and those of the Polish authors may be simply due to the fact that hatching does not form a very definite stage. In any case, lipid metabolism is shown to be quantitatively less important than that of the carbohydrates at least in early stages.

Finally, there are some data on the *breakdown of proteins*. Bialaszewicz and Mincovna found a very low but relatively constant excretion of nitrogen up to hatching. At this time it increased sharply, an evidence of the combustion of proteins. Brachet has carried out a series of determinations of ammonia and urea excreted by the egg from fertilization to neurulation. Very low values—much smaller than those of the Polish authors—were found. Perhaps a fraction of the nitrogenous wastes may be eliminated in a form other than those studied. In any case, the nitrogen excreted is small and constant in stages of fundamental morphogenesis. These conclusions are limited only to the catabolism of proteins and we will have occasion in a following chapter to examine studies on their *anabolism*. Here we will only add that the amphibian embryo must necessarily synthesize important proteins during development, this synthesis being evidently at the expense of the yolk which must first be hydrolyzed (Parnas and Krasinska).

The nitrogen metabolism of the egg of *Rana pipiens* during development was studied in serial fashion by Gregg and Ballentine. Their results show no important changes in the different fractions

studied until very advanced stages. The total nitrogen remains constant until hatching, but the nonprotein nitrogen decreases by 12 per cent during cleavage and gastrulation, rising again to attain, at the time of hatching, a value of 125 per cent of the initial amount. The soluble protein nitrogen remains constant up to the advanced neurula stage and increases slightly just before hatching, while the ultracentrifugeable nitrogen (nitrogen of the microsomes) does not vary. The soluble proteins are rich in acid groups and low in basic groups, with no variations during development. Gregg and Ballentine emphasize the fact that their methods do not permit them to detect possible changes in the nature of the proteins in different regions of the egg if these changes are not extensive enough to modify the over-all picture.

The yolk, as shown by McClendon, Jenkinson, Fauré-Fremiet and Dragoiu, is chiefly protein in nature although it probably also contains lipids. According to Fauré-Fremiet and Dragoiu, one can distinguish, on a chemical basis, a fraction soluble in alkalis and rich in phosphorus: this is probably a mixture of phospho- and nucleoproteins (personal observations). The residue after alkaline extraction appears to be keratins with a high sulfur content.

Investigators who have followed the utilization of the yolk by cytochemical methods are of the opinion that first of all a hydrolysis of the complex lipoprotein takes place. This process begins at neurulation, according to Konopacki and Konopacka, while Voss believes that it begins during cleavage and is characterized by the liberation of lipids of the plasmalogen type. A number of observations indicate that the utilization of the yolk is directly coupled with morphogenesis. Konopacki and Konopacka noted an early disappearance of basophilia of the yolk in the head region and in the nervous system; also the breakdown of the yolk granules is most rapid in the axial organs (Bragg, Ivanov). Whenever development is inhibited, the utilization of yolk is retarded. The same phenomenon is also obtained in eggs raised at supra-maximal temperatures (Hoadley) and haploid embryos (G. Hertwig, Porter). The intensive utilization of yolk would hardly agree with the low nitrogen excretion which we have just discussed, so only a small amount of deutoplasm undergoes combustion, most of it being transformed into the specific proteins of each embryonic organ.

*b. pH and Inorganic Constituents*

Some attempts have been made to measure the pH of intact amphibian eggs by the introduction of microelectrodes. Buytendyk and Woerdeman made use of the antimony electrode which involves errors not suspected at the time the observations were made. They found that the pH of ovarian eggs is about 7.2; fertilization increases the pH to 8.5, while during cleavage it gradually falls to 7.7. The blastocoel fluid is strongly alkaline (8.5). This last conclusion has

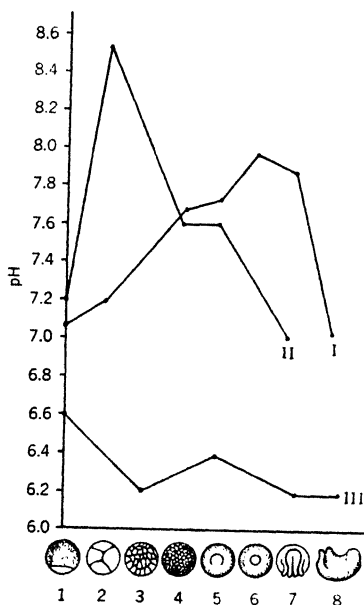


Fig. 81. Variation in pH of frog egg during development: comparison between the values of Buytendyk and Woerdeman (II), and those of Dorfman (I); (III) measurements carried out on egg breis (Dorfman).

recently been confirmed by Gregg and Ballentine who obtained a pH value between 8 and 8.4 for the blastocoel fluid; this fluid contains some bicarbonate but no protein in *Rana pipiens*. Gastrulation, according to Buytendyk and Woerdeman, does not affect pH but at the end of this stage a marked difference between the ectoderm and the endoderm is found, the former being more alkaline (pH 8.1) than the latter (pH 7.6). The figures obtained on neurulae

are as follows: ectoderm 6.9–7, nervous systems 6.8, archenteron 8.1, and endodermal mass containing yolk 6.9–7. Thus the egg becomes more alkaline at fertilization and returns subsequently to neutrality, although the blastocoel and archenteron fluids are strongly basic.

However, Dorfman made use of a hydrogen microelectrode of his own design and arrived at completely different results (Fig. 81). According to him, the pH increases progressively up to gastrulation, going from 7.06 to 7.96, falls after neurulation, and finally returns to the initial value at the time of the formation of the tailbud. Dorfman's figures are averages of measurements made at different points on the egg. This author plans to return later to the interesting question of possible differences in pH in various regions of the embryo. Dorfman and Grodensky have already shown that the pH is quite different at the two poles of the unfertilized egg, finding an average of 6.43 for the vegetal pole as against 7.07 for the animal pole. Dorfman makes the comment that his pH determinations do not confirm the findings of Trifonova and her collaborators, who believed they had demonstrated a cyclic production of lactic acid during development. However, a progressive increase in the alkaline reserve of the egg could mask the momentary accumulation of lactic acid. Such an increase was first shown in fish by Manery, Warbritton and Irving, then by Irving and Manery.

Putting aside for the moment the research of Dorfman on the  $rH_2$  of the frog's egg—work whose interest is more directly related to the study of anaerobic metabolism in the embryo—let us conclude with a few words on the metabolism of *water* and of *salts*. It has long been known (Davenport, Schaper, Fauré-Fremiet and Dragoiu) that the egg takes in considerable water during development and that this factor plays an essential role in the growth of the tadpole. Here are some figures taken from a recent publication by Ranzi, who determined the amount of water, the dry weight, and the ash of axolotl eggs in successive stages. The wet weight increases during development from 2.65 to 15.07 mg., while the organic materials drop from 2.14 to 1.67 mg. The ash increases from 0.11 to 0.15 mg. After a temporary drop correlated with the loss of the perivitelline fluid (Krogh, Schmidt-Nielsen and Zeuthen, Richards), the ash remains constant for nine days. Water makes up 53.8 per cent of the fertilized egg, 60.6 per cent of the blastula, and 66.5 per cent of the gastrula. This increase is parallel with the increase in volume during

cleavage (14.5 per cent) and gastrulation (7.6 per cent), according to Bialaszewicz. Thus, one must conclude that even in early stages the growth of the egg depends on hydration. This increase in percentage of water has as a consequence a decrease in density which goes from 1.106 to 1.048 (Briggs) during the first week, this drop being broken by a temporary rise at the time when the archenteron becomes smaller (near the end of neurulation). This temporary rise in density of the embryo has been confirmed by Brown and is a result of the expulsion of the archenteron fluid. Finally, Ranzi and Arosio have reported an increase in certain metals in the tadpole after hatching (Al, Ba, Ca, Cu, Fe, K, Mg, Mn, Na). All the evidence points to the conclusion that these ions are derived from the mucous jelly.

### *c. Enzymes and Reducing Substances*

The only systematic study of the enzymes of the frog's egg was carried out in 1907 by Herlitzka and the techniques used appear rather crude today. Nevertheless, let us go over the general conclusions of Herlitzka. The fertilized egg contains indophenol oxidase and catalase while it possesses no tyrosinase nor peroxidase; amylase was also found as well as invertase, which transform glycogen into glucose. The latter substance was not attacked by the eggs treated according to Herlitzka's method. The appearance of peroxidase coincided with the formation of haemoglobin while tyrosinase appeared in the ten-day-old embryo. Spirito (1934, 1935) has confirmed the presence of a weak peroxidase reaction in amphibian eggs, localized near the animal pole. In the toad, however, the entire egg becomes intensely blue in the presence of benzidine and peroxide. Slonimski (1931) has verified that the anlagen of the red blood cells (blood islands) give a strong peroxidase reaction. According to Spirito, it is caused by a true thermolabile peroxidase, which appears just before haemoglobin. In addition, the lamprey egg, like that of the toad, shows a strong reaction, while teleost embryos contain only a small amount of peroxidase, sharply localized in the blastodisk.

Brachet has also studied some of the respiratory enzymes present in the egg of *Rana fusca*. The indophenol and peroxidase reactions are negative in the whole egg and in the isolated cells of blastula and gastrula. When Nadi reagent is added to a brei of eggs one sees a

distinct inhibition of the spontaneous autoxidation of this substance, signifying that the egg contains reducing systems capable of decolorizing indophenol blue as fast as it is formed. It is thus impossible to draw any unequivocal conclusions about the indophenol oxidase reaction in the frog's egg. The negative reaction furnished by this material does not prove that the enzyme is lacking in the egg, however. On the contrary, the fact that respiration is very cyanide-sensitive demonstrates that oxidation is catalyzed by an enzyme resembling indophenoloxidase. This example shows once again how necessary it is to be careful with cytochemical reactions designed to detect the oxidases.

A brei of frog eggs shows increased respiration, as illustrated by Figure 82. This is a very common phenomenon which has been

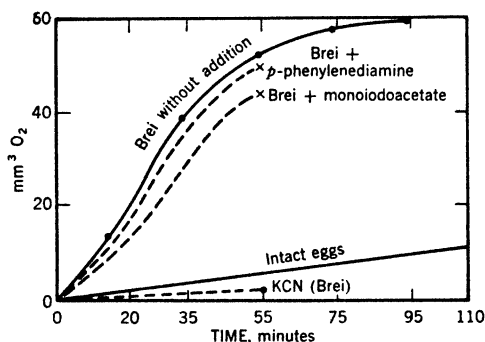


Fig. 82. Effect of cytolysis on oxidations in frog egg; effects of cyanide, monoiodoacetate, and of paraphenylenediamine (J. Brachet).

described for *Sabellaria* (Fauré-Fremiet), *Arbacia* (Tang), *Melanoplus* (Bodine and Boell), etc. The oxygen consumption exhibited by the brei is inhibited by cyanide, although monoiodoacetate and paraphenylenediamine do not influence it. One must conclude that the fertilized egg contains an amount of respiratory enzyme sufficient to catalyze much higher rates of oxidation than normal. Normally the enzyme is probably far from being saturated by its substrates. This interpretation is the more reasonable since a brei of tadpoles, which have a considerably higher metabolism than fertilized eggs, has a lower rate of oxygen consumption. It is possible that the increase in oxidation which parallels development results simply

from the progressive saturation of the respiratory enzyme without any synthesis of the latter. Possibly this saturation is a result of changes in cytoplasmic structure, conforming with Runnström's hypothesis for the sea urchin (see Chapter IV). One may also envisage, by analogy with the case of the sea urchin egg a progressive liberation of cytochrome or an activation of the dehydrogenases. The few attempts made to verify this hypothesis have not led to conclusive results. Centrifugation, which profoundly changes the distribution of fats, glycogen, and yolk, has hardly any effect on the rate of oxidations. Cytochrome appears to be lacking in the amphibian egg, while ability to reduce methylene blue shows hardly any variation from stage to stage. Results and conclusions similar to those of Brachet were obtained by Spiegelman and Steinbach in *Rana pipiens*. In this species, too, oxygen consumption increases when early embryonic stages are homogenized and diminishes when older embryos are so treated. The oxygen consumption of these breis is inhibited by cyanide, but sodium azide, which strongly inhibits the respiration of intact eggs, has hardly any effect. The amount of cytochrome oxidase increases during development, while cytochrome c is not a limiting factor in respiration. The authors conclude that it is the spatial relations between the enzymes and the substrates which determine the rate of oxygen consumption.

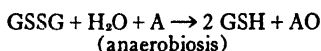
However, determinations of cytochrome oxidase and succinic dehydrogenase by Boell on *Ambystoma* show that these two enzymes are synthesized during development, following an exponential curve that is parallel to the increase in oxygen consumption. It is not, however, the amount of cytochrome oxidase that limits the absolute rate of the oxygen consumption. The synthesis of cytochrome oxidase and of succinic dehydrogenase represents, according to Boell, the synthesis of metabolically active cytoplasm at the expense of the yolk. It probably corresponds to the increase in nucleoprotein granules, since it is to these that the two enzymes are bound (Stern, Chantrenne).

The amount of glutathione in the frog's egg remains unchanged up to hatching (Kamiya; J. Brachet 1934). On the other hand, there is a synthesis of ascorbic acid (Suomalainen) which appears to accelerate development slightly (Slonimski, 1938). Finally, it may be noted that amphibian eggs contain some auxins in small amounts (Rose and Berrier). They appear relatively late and it is doubtful if they play any role in mitosis.

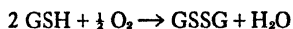
*d. Anaerobic Metabolism of the Frog Egg*

We know that the frog egg is able to undergo cleavage in the absence of oxygen, but what are the sources of energy at its disposal under these conditions? One may think of a fermentation forming lactic acid from glycogen (glycolysis) as in muscle, or a second alternative would be the continuation of normal oxidation at the expense of an oxidative reserve which is used little by little in anaerobiosis. Since we have seen above that lactic acid production increases only slightly when the egg is placed under anaerobic conditions, the possibility of an oxidative reserve appears more likely.

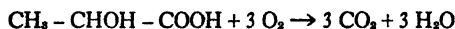
Brachet devoted a series of experiments (1934) to the elucidation of the phenomenon that occurs when eggs are deprived of oxygen for a few hours. When eggs treated in this way are placed back in air they undergo an increased respiration for some time, a phenomenon well known for muscle and for nerve. Now during the anaerobic phase the egg contracts an *oxygen debt* which it "pays" when oxygen is readmitted. This debt has two possible interpretations. Either there is an accumulation of waste products of anaerobic metabolism, such as lactic acid, which is rapidly oxidized on return to air, or the oxygen debt constitutes an oxidative reserve which is expended during the anaerobic phase. One may imagine for example, that this reserve consists of oxidized glutathione which in becoming reduced oxidizes some substance A according to the reaction:



In air, the oxidative reserve is regenerated:



This reaction differs from the oxidation of lactic acid which may be written as follows:



Actually, in this case  $\text{CO}_2$  is produced and the respiratory quotient will be about unity, but when there is a simple reconstitution of an oxidative reserve we have an autoxidation without the formation of  $\text{CO}_2$ .

The question can be settled by measuring the respiratory quotient at the time when the egg pays its oxygen debt. Our measurements gave us values varying between 0.27 and 0.52, leading to the conclusion that the oxygen debt consists chiefly of an autoxidation rather than an oxidation of the lactic acid type. Thus the frog's



egg is more comparable to nerve than muscle with regard to anaerobic metabolism. It is very probable that when eggs are placed under anaerobic conditions their oxidation continues at the expense of an oxidative reserve, and when this is expended, their glycolysis then increases. A similar interpretation would explain Lennerstrand's failure to find an appreciable increase in glycolysis when eggs are deprived of oxygen for seven to eight hours, although the amount of lactic acid greatly increases when anaerobiosis continues for twenty hours.

We have also found that the frog's egg continues to produce  $\text{CO}_2$  when placed in nitrogen, this  $\text{CO}_2$  originating principally from the continuation of oxidation at the expense of an oxidative reserve. A smaller fraction results from the reaction of lactic acid with the bicarbonates of the egg. This anaerobic formation of  $\text{CO}_2$  has been confirmed by Latinik-Vetulani, who found that it was weak at the beginning of cleavage, increased in the blastula and gastrula and fell off at the end of gastrulation, and finally increased sharply at neurulation, falling off once more later. We have seen (Fig. 62, Chapter VII) that Latinik-Vetulani's curve recalls that obtained by Trifonova and her collaborators for glycolysis, although this parallel is not exact.

The problem has been re-examined by Spirito and his students on a comparative basis. It will be remembered that the eggs of the toad and of *Discoglossus* are more resistant to lack of oxygen than those of the frog, *Hyla*, and axolotl. Spirito (1938) measured anaerobic  $\text{CO}_2$  production by means of a colorimetric method in *Bufo*, *Rana agilis*, and *Rana esculenta*, and noted that it is decidedly greater in *Bufo*,—the species in which eggs develop best in absence of air. According to Spirito, no nonvolatile acid is produced under anaerobic conditions and he concludes that the amphibian eggs that he studied do not produce lactic acid. This conclusion is very dubious since the method used by the Italian author would detect only the lactic acid which diffuses into the medium. It is much more probable that the small amounts of this acid which appear during glycolysis are immediately bound by the alkaline reserve of the egg. The reality of anaerobic glycolysis, however small, is shown by all of the experiments in which lactic acid was determined directly. Spirito, in his paper, after having criticized Brachet's experiments at length, came to the same conclusion: that anaerobic development in the frog's egg is at the expense of an oxidative reserve, which will be greatest in those forms best supporting anaerobiosis.

In regard to the egg of *Discoglossus*, which is particularly well adapted to lack of oxygen, Spirito (1939), however, found only a very weak oxidative reserve. Nevertheless, this egg is capable of an appreciable glycolysis, as the determinations of lactic acid have demonstrated. Thus it appears that two mechanisms, oxidative reserve and glycolysis, coexist, but with a preponderance of one or the other depending on the species.

Two of Spirito's students, Friggeri and Giolitti, attempted to determine the chemical nature of this oxidative reserve. In Brachet's paper two possibilities, suggested respectively by J. Needham and L. Rapkine, were noted. Rapkine drew attention to the possibility that oxidized glutathione may be the oxidative reserve, and Needham put forth the hypothesis that the oxygen utilized by the egg during anaerobiosis comes from hydrogen peroxide which is decomposed by catalase. It was just these two possible explanations that the Italian authors examined. The presence of catalase in the amphibian egg has been known for a long time (Herlitzka, Bialaszewicz and Mincovna, etc.). Friggeri (1940) reported that there was a satisfactory parallel between the amount of catalase in amphibian eggs and their resistance to anaerobiosis, the toad neurula containing six times more catalase than that of the frog, while *Discoglossus* occupied an intermediate position. In addition, Friggeri (1941) determined the catalase activity in neurulae raised at various temperatures, Spirito having observed that development proceeds further in absence of oxygen at 10° than at 20°. The eggs which differentiate at the lower temperature were actually richer in the enzyme but only by 7 to 13 per cent.

Giolitti determined the amount of —SH and —SS— groups in toad neurulae, finding that embryos which developed at 20° contained only —SH radicals while those raised at 10° had 50 per cent —SS— and 50 per cent —SH. Thus, larvae which showed more resistance to anaerobiosis had, as one might suppose, the higher concentration of —SS— radicals, which may form part of the oxidative reserve. More recently (1943), Giolitti showed that the proportion of —SH to —SS— groups in the egg varies with different species, the —SS— radicals being more abundant as resistance to anaerobiosis increases.

In conclusion it may be noted that Dorfman, as a result of some determinations on the  $rH_2$  of the frog's egg during anaerobiosis, raised considerable doubt regarding the reality of an oxidative re-

serve in this species. The values he obtained appear to him too low to assume the presence of a high redox potential system capable of playing the role of an oxidative reserve. In addition, the anaerobic  $rH_2$  of the egg does not change during its whole developmental period, although its susceptibility to lack of oxygen increases continuously. However, one can answer this criticism by noting that the rapid blockage of development of advanced stages in anaerobiosis is easily understood by reason of the continuous increase in the level of oxidation during development. The neurula consumes three to four times as much oxygen as the morula, so it is easy to see that it would use up its oxidative reserve more rapidly.

In regard to the low anaerobic  $rH_2$  of the egg one wonders whether some systems of high potential are not localized in the regions of the formed structures of the cytoplasm. Brachet observed (1934) that pigment granules will reoxidize reduced methylene blue during anaerobiosis. Their  $rH_2$  should therefore be greater than fourteen, although Dorfman found values lower than this. We may thus conceive of an oxidative reserve bound to the microscopic or submicroscopic structures in the cytoplasm. A microelectrode of large area will obviously not detect these small localized regions.

Recalling the interesting work of Dorfman showing no differences in  $rH_2$  at the two poles of the egg, this observation makes it likely that there is a homogenous distribution of the principal redox system in the egg. If variations in metabolism are found along the animal-vegetal axis then we may assume that they are of a quantitative rather than qualitative nature. This will be considered in the following chapter.

Before closing this discussion on the anaerobic metabolism of the frog's egg, it is worth recalling that Barth and Jaeger have demonstrated the presence of a labile phosphate ester in the frog's egg, probably identical with adenosine triphosphate. This ester is hydrolyzed under anaerobic conditions with the liberation of inorganic phosphate and is resynthesized under aerobic conditions. It may well be, as Barth and Jaeger suggest, that continuation of development during anaerobiosis is made possible by the breakdown of an energy-rich phosphate ester which is regenerated in the presence of oxygen through coupling with the oxidations. Such an explanation has the advantage of fitting in perfectly with current ideas about the chemical mechanism of muscular contraction and sperm

motility. In addition, according to Barth and Jaeger, only part of the inorganic phosphate liberated during anaerobiosis comes from the labile phosphate ester.

### 3. Conclusions

Of the facts just considered, there is one that stands out particularly. This is the abrupt change in metabolism at gastrulation, the time when we find a sudden change in R.Q. and when glycogenolysis begins. Glycolysis also increases as if a complete carbohydrate catabolism suddenly became predominant. Are these changes in metabolism coupled with the morphogenetic movements characteristic of gastrulation? It would be premature to say that they are, since we really lack too much data on species other than *Rana fusca*. We must know, for example, whether the low respiratory quotient, indicating incomplete oxidation, is really characteristic of all amphibian eggs during cleavage and, in addition, whether the beginning of carbohydrate metabolism always coincides with gastrulation. We shall have occasion to discuss at length the coupling between carbohydrate oxidation and the beginning of morphogenesis, properly speaking. In any case it has now been established that the embryo, up to hatching at least, preferentially utilizes carbohydrates.

Another point is worth mentioning: that is, that the progressive hydration of the embryo conditions growth much more than cellular division. Does this hydration have any real morphogenetic role? Does it participate in gastrular movements? We cannot answer this question with any certainty and we will discuss later the evidence at our disposal in this respect.

We know that the amphibian egg is a facultative anaerobe during segmentation, but as development progresses this resistance to lack of oxygen decreases, which is simply the result of increasing energy demands during ontogenesis. The oxidative reserve is probably more rapidly expended as the egg reaches more advanced stages. Glycolysis appears in general to be a less important factor than the continuation of oxidation at the expense of an oxidative reserve. There is also some reason to believe that catalase and —SS— groups take part in the anaerobic respiration of the egg. Gross differences exist between species in regard to the metabolism of amphibian embryos in absence of oxygen, and the zoological interest of these differences will no doubt become apparent some day.

Finally, measurements of pH and  $rH_2$  of the egg bring to light a heterogeneity along the animal vegetal axis. This is a matter of continuous physicochemical differences, in gradient form, apparently only quantitative in nature.

The facts gathered on the metabolism of the frog's egg form a promising point of departure for future research. We shall shortly see that they have already contributed to our knowledge of the mechanics of morphogenesis.

## CHAPTER X

# Metabolism of the Organization Center and Chemical Nature of the Inducing Substance

### 1. General Considerations

We know that the nervous system of vertebrates does not differentiate autonomously; it is the end result of an *induction* by the underlying cells which constitute the organizer. Let us review briefly some of the fundamental facts.

The topography of the various presumptive regions of the young gastrula is rather precisely known thanks to the work of Vogt, Pasteels, etc., who have chiefly made use of the ingenious method of vital staining. This method consists of staining vitally a definite region of the young gastrula, making it thenceforth easy to follow the ultimate fate of the cells thus marked. This technique has the double advantage of showing the plan of the primordia and of revealing the nature of the gastrular movements. Figure 83 shows the position of the principal primordia in the young urodele gastrula.

As we see in Figure 84, taken from Spemann, the location of the different areas changes profoundly during gastrulation. The chordamesoderm (in fine lines on the diagram) invaginates completely, as does the entoderm (in white). On the dorsal side, the notochord material is in direct contact with the presumptive nervous system, and forms the roof of a cavity, the archenteron, which is centrally located. The cavity of the blastocoel is eventually obliterated.

The following experiments very quickly demonstrate the importance of the notochordal material for the formation of the nervous system. (*a*) If a small piece of presumptive epidermis is exchanged for an equivalent sized piece of presumptive neural plate in the young

gastrula, a perfectly normal embryo develops (Spemann). Thus the presumptive nervous system is not "determined" at this stage but differentiates according to the position to which it is transplanted. (b) If we explant a fragment of presumptive epidermis and

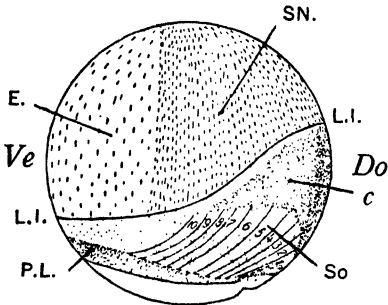


Fig. 83. Map of presumptive areas in young gastrula of *Triton*: L. I., limit of invagination; c, notochord; SN., nervous system; E., ectoderm; So, somites; P.L., lateral plate; Ve, ventral; Do, dorsal (Vogt).

one of presumptive neural plate and culture them *in vitro* in Holtfreter's solution, both of them develop in the same way and give rise to somewhat abnormal epidermis. In particular, the piece of presumptive neural plate will *not* differentiate into nervous system. But if to these explants a piece of the dorsal lip of the blastopore is

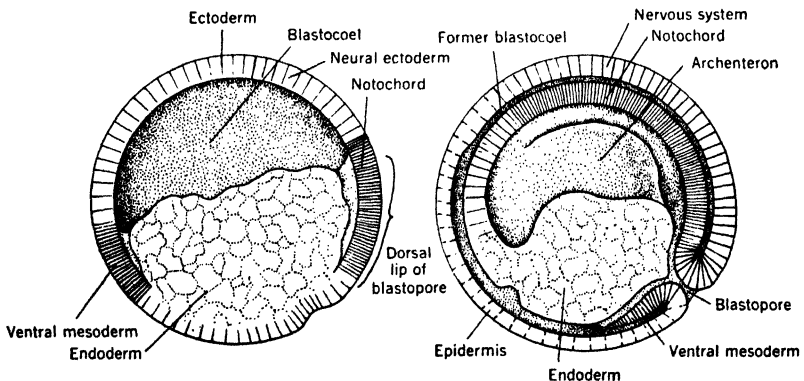


Fig. 84. Sections through a young and an advanced gastrula (diagrammatic sketch after Spemann).

added, *both of them* give rise to a nervous system. Thus, the dorsal lip of the blastopore exercises a morphogenetic influence which may act on the presumptive epidermis as well as the presumptive neural plate, behaving like an "organizer," that is, capable of *inducing* a

nervous system. (c) If a dorsal lip is grafted into the ventral half of the gastrula (Spemann and H. Mangold), it will invaginate and become located under the ventral presumptive epidermis of the host, there inducing a secondary embryo (Fig. 85). Thus the organizer is localized in the dorsal lip of the blastopore of the young gastrula stage, and at the end of gastrulation it is in the roof of the archenteron. It thus corresponds to the future notochord and prechordal endoderm. Under the influence of the organizer the nervous system will form by means of an inductive action.

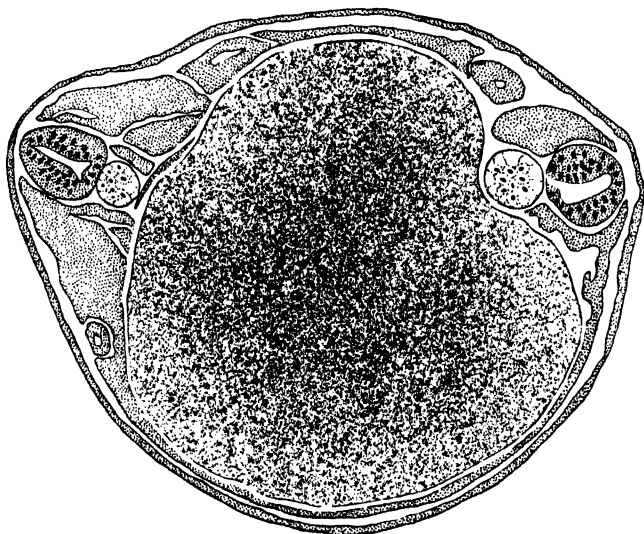


Fig. 85. Double embryo obtained by a graft of the organizer into the ventral half of a gastrula: *left*, nervous system, notochord, and somites of the host; *right*, an induced secondary embryo (Spemann and Mangold).

We now know that neural induction is active in all of the vertebrates, and it has also been established that many other organs in addition to the nervous system originate by induction. This is demonstrated in a particularly striking fashion by the induction of the lens of the eye by the optic vesicle (Spemann). Here we shall confine ourselves to a discussion of the induction of the nervous system and say only a few words concerning the formation of sensory structures. Since the efforts of the chemical embryologist have been concentrated on the primary organizer of the nervous system it may be asked what is known concerning its nature.



In 1928, Child attempted to include the organizer concept in his theory of physiological gradients. As we have seen earlier, he conceives of a primary gradient decreasing from animal to vegetal pole, which is replaced at gastrulation by a new physiological gradient whose most active region would be none other than the organizer. Child's hypothesis stems from experiments by Bellamy, who found an increased susceptibility of the organizer toward a number of chemical compounds (see Chapter IX), and it has found fresh support in the work of Gilchrist. This investigator placed amphibian eggs in a "thermal gradient," applied in such a manner as to heat the ventral side and cool the dorsal half. In a few cases, he obtained a secondary embryo on the ventral side where the metabolism was increased by heat. We will have occasion to discuss these facts shortly. A. Brachet (1931) came to a conclusion similar to that of Child and considered the organizer as a kind of activator, wherein there is an intense metabolism, the energy from which may be transmitted to neighboring regions.

At the same time, Marx demonstrated that the organizer, after treatment with narcotics, retained its inducing ability. He did not investigate whether the metabolism was lowered but it is probable that such was the case. This observation cast doubt upon the "metabolic" theory of the organizer, and the observations of Bautzmann, Holtfreter, Mangold and Spemann which had great repercussions at the time of the publication (1932) appeared to invalidate the theory completely. The German embryologists established decisively that the *dead organizer* can function as an inductor, so it was necessary to admit that the agent responsible for the induction was a *chemical substance* present in the organizer. But if one really thinks about it, nothing is apparent in the experiment cited above which denies that this active substance arises from intense metabolism, and neither may one exclude a gradient distribution of the substance.

This is why Huxley and de Beer, in their very original book, did not hesitate to explain morphogenesis in terms of a metabolic gradient, although their arguments are, to be sure, not always convincing. Dalcq and Pasteels prefer a gradient in terms of the distribution of the constituents of the egg to the metabolic gradient of Child, and thus return to the original concept of Boveri, at least in regard to the nature of the gradient. We owe to Dalcq and Pasteels a remark-

able attempt at a general explanation of embryonic development in the most diverse forms. One of the factors upon which morphogenesis would be based, according to this view, is the animal-vegetal gradient revealed by the progressive size increase of the yolk granules (Fig. 86). This is a *vitelline gradient* with the most active region at the vegetal pole. The egg would also show differences in a dorso-ventral direction, these differences taking the form of a *field* with the focal point situated dorsally. Morphogenesis would then result from the interaction of the dorsal field and the vitelline gradient,

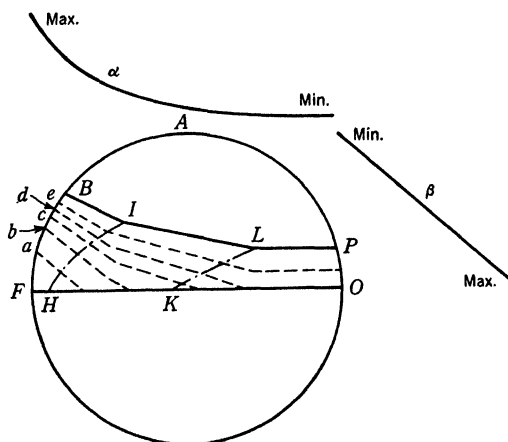


Fig. 86. Schematic representation of the theory of Dalcq and Pasteels. Anuran blastula: F = beginning of the cortical dorsal field with a decreasing intensity following the curve  $\alpha$ ; curve  $\beta$  corresponds to the decrease in vitelline gradient with its maximum at the vegetal pole. The lines a, b, c, d, e are lines of the same concentration of CV during gastrulation. The ratio C/V is the same on all points on lines HI and KL (after Dalcq and Pasteels).

there being a chemical combination postulated between a compound C of the dorsal field and a vegetative substance V. The concentration of the product CV would then determine the different regions in the egg. The greater the amount of CV in any region, the greater its "morphogenetic potential" will be. For example the concentration of CV in the notochord would be greater than that of the somites, which would themselves have the advantage in this respect over the intermediate mesoderm. The latter would in turn be richer in CV than the ventral mesoderm which would have the lowest morphogenetic potential in the chorda-mesoderm layer. In order to explain

the segregation of the various primordia, Dalcq and Pasteels invoke the idea of a series of *thresholds*. In the chorda-mesoderm, as we have seen, the amount of CV would decrease dorsoventrally in a regular fashion. When this concentration exceeded a certain value  $S_1$ , notochord would be formed; if it fell below  $S_1$  but still remained above a second value  $S_2$ , somites would differentiate. Similarly between  $S_2$  and  $S_3$  (where  $S_3 < S_2$ ) there would be intermediate mesoderm. We cannot enter into the details of this theory which has the advantage of explaining gastrulation as an organ-forming differentiation. First applied to the amphibian egg, it has been extended readily to other vertebrates, the ascidians and the sea urchin. Various types of experiments (rotation of the fertilized eggs, centrifugation by Pasteels, translocations by Dalcq) have lent support to the theory. Let us emphasize chiefly that Dalcq and Pasteels attributed morphogenesis to a reaction between the vitelline gradient and a dorsal field with a cortical localization. It is this reaction which would give rise to the organizer substance (organisin).

This concept as yet appears to be merely an interesting attempt to explain morphogenesis. Certainly the facts revealed by Dalcq and by Pasteels are often open to other interpretations. Rotmann has recently published a critical discussion of this theory, which we have referred to here especially because it will be convenient to consider it in relation to biochemical evidence. It is with this same intention that we may pause to consider certain results of F. E. Lehmann, who thinks that the layer of cytoplasm just under the cortex of the fertilized egg plays a very important role. This *subcortical plasma* constitutes, in axolotl, a marginal ring which tends to be thicker on the dorsal side (Fig. 87). When the egg is rotated 180°, the cortical plasma is disturbed and shows a new distribution, which would explain the appearance of a second blastoporal groove, followed by a secondary embryo in rotated eggs. The subcortical plasma later goes to the dorsal lip of the blastopore and finally into the roof of the archenteron: it is thus one of the components of the organizer and as Lehmann suggested, it would be very valuable to study it cytochemically.

In conclusion, let us mention some observations by Barth which, if confirmed, modify radically our ideas about the organizer. We have seen above that a fragment of presumptive epidermis or neural plate cannot form a nervous system in the absence of the organizer

(Holtfreter, Raunich). But Barth claims to have obtained *nervous systems without organizer action*: it is necessary to maintain the dorsoventral polarity of the explant by placing it in a narrow trough hollowed out of agar. The same result may also be attained by placing two fragments together end to end and taking care to maintain their polarity. Neuralization is more frequent in fragments taken from the presumptive neural plate than from the epidermis. These facts led Barth to attribute an important role to the dorsoventral gradient in the epidermis. These results are quite opposed to current ideas and it was fortunate that Holtfreter undertook a repetition of the experiments in order to clarify the situation. Holtfreter came to the conclusion that neural differentiation of epidermal explants in the absence of the inductor was found in only one species,

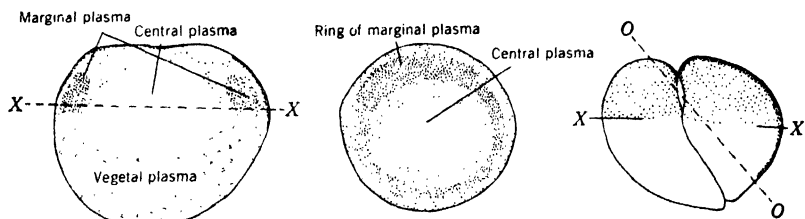


Fig. 87. Distribution of the different plasmas in the egg of axolotl: *left*, median section; *center*, section just above the equator, XX; *right*, displacement of the marginal plasma after reversal of the egg; the lower limit of the plasma goes from OO to XX (F. E. Lehmann).

*Amblystoma punctatum*; in this species the formation of nervous systems in ectoderm cultivated *in vitro* seemed to be caused by the toxicity of the saline solution. In fact, there was a correlation between the frequency of neural reactions and the number of cells showing cytolysis. The same correlation is found when one considers the localization of the nervous system in relation to the necrotic cells. This explanation explains Barth's results satisfactorily, since we shall see later that the cytolysis of certain cells can liberate substances capable of inducing a nervous system in the ectoderm. In addition, Holtfreter did not find differences in the frequency of neurulations in the presumptive epidermis and presumptive neural plate of young gastrulae of *Amblystoma*, but in more advanced gastrulae, the presumptive neural plate showed a greater tendency toward neural differentiation than did the epidermis. A later study by Holtfreter

further supports this approach, for by cultivating explants in abnormal salt solutions (weakly alkaline, calcium free, etc.) he could deliberately stimulate cytolysis and found that the frequency of neural reactions in the epidermis is proportional to the number of cells affected.

All the facts presented thus far cannot be understood unless we suppose the existence of an organizer substance. They also furnish evidence in favor of animal-vegetal and dorsoventral *gradients* without, however, deciding whether these gradients consist in variations in metabolism or in a heterogeneity of cytoplasmic constituents. It is the task of chemical embryology to find the answer to this question. Let us now see how far it has progressed.

## 2. Cytochemical Observations

We are indebted to Voss (1931) for the first information of a cytochemical nature on the organizer, his observation concerning *plasmalogen*—a phosphatide. Voss fixed axolotl gastrulae with sublimate, immersed them in decolorized fuchsin, and, after washing, embedded the embryos in paraffin and sectioned them. He found that only the external layer became colored, and attributed this situation to the more intense oxidations in the cortex which transformed certain lipids into plasmalogen. In the gastrula Voss noted that the invaginated cells lost their plasmalogen (Fig. 88), but Brachet believes that this is an artefact resulting from insufficient penetration of the reagents. He has repeatedly carried out the plasmal reaction on amphibian eggs cut in half after fixation with sublimate and has found that the whole surface of the section becomes homogeneously colored.

Some recent observations by Holtfreter (1946) on the distribution of plasmalogen agree with those of Brachet in showing that this phosphatide has a more general distribution than thought by Voss. Nevertheless the observations of Voss are not without interest. He noted, for example, that the plasmalogen appears in close contact with the yolk, probably as a result of a dissociation of a complex of the deutoplasmic granules. Similar facts were reported earlier by Konopacki and Konopacka. Voss concluded that the inducing substance arises through a disintegration of the yolk; this deduction becomes more interesting in connection with the ideas of Dalcq and Pasteels on the morphogenetic role of the yolk.

The distribution of *glycogen* during gastrulation has also excited keen interest. Woerdeman (1933) detected this polysaccharide in the usual manner: fixation in alcohol or Carnoy's fluid, and staining with Lugol's solution or Best's carmine controlled by digestion with salivary enzymes. Woerdeman reported that the animal half of the egg contained more glycogen, the substance being found in greatest amount in the cells nearest the blastocoel, which latter also contained a little as did the perivitelline fluid. These

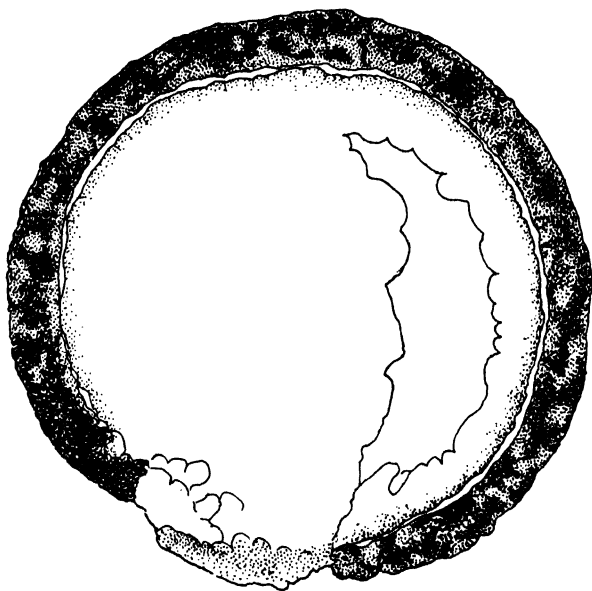


Fig. 88. Localization of plasmalogen in gastrula of axolotl; reaction becomes negative in the invaginated material (lower right) (Voss).

facts agreed very well with the earlier findings of Konopacki and Konopacka. Woerdeman also determined that it is possible to recognize two gradients in the blastula, oriented in an animal-vegetal and a dorsoventral direction, respectively. At gastrulation a clear difference is seen between the invaginated cells and those which remain on the surface, the former having lost most of their glycogen, while the latter become intensely colored. Invagination is thus accompanied by an intense *glycogenolysis*. At the end of gastrulation, the presumptive epidermis and the neural plate are rich in glycogen,

the endoderm contains only traces, and the chordamesoderm contains only small amounts. In the neural plate there is an evident cephalo-caudal gradient.

Woerdeman's results were extended by Raven (1933), who grafted an organizer in the ventral half of a gastrula. Here, too, glycogen disappeared during invagination, the cells of the host and the graft being affected simultaneously. This is evidence, according to Raven, of a coupling between glycogenolysis and invagination. Raven (1935) continued his experiments by grafting a fragment of the presumptive epidermis into the lip of the blastopore, where it is then drawn in by gastrular movements and invaginates. The author claims that it loses its glycogen just like the neighboring cells of the dorsal lip. This epidermal fragment also acquires the ability to induce following its contact with the organizer and thus there appears to be a relation between glycogenolysis and induction. The counterpart of this experiment consisted in implanting epidermis into the blastocoel cavity. In this case it did not invaginate and the glycogen did not break down.

The facts described by Woerdeman in axolotl have also been found by Tanaka in the toad, frog, and *Triturus pyrrhogaster*, and by Brachet in *Discoglossus* (1935). Tanaka drew attention to the fact that glycogenolysis is also found in the region of the ventral lip, although these cells are devoid of inducing activity. In addition, the amount of glycogen in the entire egg decreases during gastrulation. Thus, glycogenolysis is not a specific characteristic of the dorsal lip of the blastopore and it becomes doubtful if it plays a decisive role in induction.

Pasteels arrived at even more negative conclusions with regard to the role of glycogen. After a comparative study of a series of fixatives he doubts that Carnoy and alcohol can be used satisfactorily, since the amount of glycogen in teleost embryos appears very variable according to the fixative used. Pasteels obtained good preservation of glycogen *in situ* only if picric dioxane was used as a fixative. Under these conditions, he found no glycogenolysis during invagination in the trout and no glycogen in the embryos of birds and mammals. This latter point is confirmed by biochemical analysis (J. Needham, 1931). As a consequence, glycogenolysis can scarcely have general significance, particularly since the phenomenon is lacking in other vertebrates which also form nervous systems by means

of induction. It then became necessary to verify Woerdeman's observations, and Pasteels began to do so immediately. Studying the gastrulae of axolotl, toad and frog fixed in Allen's fluid or in picro-dioxane, he found no glycogenolysis. The earlier workers were thus victims of an artefact, since glycogen tends to shrink away from the fixing agent and its distribution may be profoundly changed. A large part of the glycogen present in the invaginated material passes into the blastocoel, thus simulating a glycogenolysis. If the latter really exists, it is too small to be detected by cytochemical means, and only quantitative determinations would permit the solution of this question.

Pasteels' results concerning the chick embryo, however, are not agreed to by Jacobson. This author claimed, contrary to classical opinion, that the epiblast cells invaginate at gastrulation to form the entoblast, and at that time lose their glycogen. However, it is not very probable that a real gastrular invagination occurs. It may also be noted that the presence of glycogen in the young chick embryo contradicts the conclusions of biochemists. In addition, according to Jacobson, the presumptive nervous system is characterized in the primitive streak stage by an increased lipid content.

The cytochemical results in relation to the lipids and glycogen thus remain rather deceptive, as we have just seen. Are we more fortunate in the case of *proteins* which present the advantage of being able to be fixed *in situ* more easily?

Numerous authors have followed microscopically the disappearance of the yolk granules during embryonic development (Saint-Hilaire, Konopacki and Konopacka, H. Hibbard, Voss, Kedrowski, Porter, Bragg, etc.). They are unanimous in stating that the utilization of yolk is appreciable only in relatively late stages. It is at the time of hatching that the phenomenon really becomes evident, and, at this time, the disappearance of yolk is directly proportional to morphogenesis, being particularly marked in the superficial layer, especially in the nervous system. In addition, it follows a cephalocaudal gradient.

It is easy to see that the egg consumes its protein yolk at the time when organs show histological differentiation; this differentiation parallels the synthesis of specific proteins which can only originate from the yolk reserve. Does this mean that utilization of yolk may not occur earlier, at the time when primary morphogenesis takes



place? Certain observations of Ivanov, carried out on favorable material, show that there is actually a very appreciable utilization of yolk as early as the second half of gastrulation in *Triton taeniatus*. Measurements carried out on the chordomesoderm at the beginning of neurulation reveal a very high yolk utilization in the notochord and the somites, although it is almost absent in the lateral and ventral mesoderm. The vitelline platelets disappear much more rapidly in the dorsal endoderm than in the other cells of this layer. According to Ivanov, yolk consumption slows down at the end of neurulation, at the tail bud stage. The Russian author concludes that the determination of the various organs is accompanied by an increased yolk utilization, the latter being especially intense when the primordium of the notochord comes in contact with the presumptive neural plate. This, of course, is exactly the time when the organizer exercises its inducing action.

We owe to Kedrowski an interesting study of the basophilic proteins of the frog embryo (1937). He found them appearing just before hatching when tissue differentiation is underway. These basophilic proteins, which Kedrowski also calls acid colloids, certainly have a vitelline origin. In the tadpole they are seen in particularly large amounts in the region of the nervous system, and in the sensory organs, the lens exhibiting an intense affinity for basic dyes. Some time after the appearance of the acid colloids the living cells of the tadpole begin to take up neutral red. Kedrowski believes that the basophilic proteins which, in the young tadpole, do not stain with neutral red are present in the form of a complex, and that there would be a liberation of the acid colloids from a complex during late development. Kedrowski attributed a physiological role to these acid colloids because he found that the development of tadpoles stained with neutral red was retarded. While the basic proteins would not be indispensable for simple maintenance of the life of the cell, they would according to Kedrowski, take part in synthesis, and for this reason, he suggested calling them *anabolites*. They are probably lipoprotein complexes rich in phosphorus. Embryonic development is subdivided by Kedrowski into a series of successive phases, presenting distinctive biochemical characteristics. In the table on p. 357 he pictures this succession.

We have had the good fortune (1938, 1940, 1941) of being able to repeat and extend the observations of Kedrowski. In material more favorable than the frog it is possible to detect the basophilic

proteins at a much earlier stage than hatching, and, in addition, we have been able to show that these acid colloids are in fact *ribonucleoproteins*, the basophilia of the embryos disappearing completely after the action of ribonuclease. The ribonucleoprotein nature of the anabolites has since been confirmed by Kedrowski (1941).

Our studies were carried out on a large number of urodeles and anurans, a few species of fish, and the chick. We have also investigated the localization of —SH groups tied to the proteins using the Giroud-Bulliard reaction (Chapter I) on embryos fixed with trichloroacetic acid. It will be recalled that under these conditions the proteins are denatured and that —SH groups which were not present in the native state appear. Thus the method used does not furnish any information regarding the —SH groups present in the living egg, but is only a procedure for the detection of certain particu-

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1	2	3	4
<i>Segmentation:</i> beginning of growth and of utilization of yolk. Increase in respiration.	<i>Morphogenesis proper:</i> increase in yolk utilization, appearance of anabolites. Increase in R.Q. and glycolysis.	<i>Differentiation:</i> disappearance of the yolk. Liberation of anabolites which change state. Modification of general metabolism. Large increase in respiration.	Animal takes on food and begins active movements.
Fertilization	Gastrulation	Hatching	Appearance of gills

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lar proteins. Ribonucleic acid was identified by its affinity for pyronine after staining with a mixture of methyl green and pyronine; in certain cases, one can substitute toluidine blue with advantage. The specificity of the stains has been subsequently verified by the use of ribonuclease and by quantitative determinations.

The two methods furnish the same results except that the nuclear sap gives a positive nitroprusside reaction although it is free of ribonucleic acid. One may thus conclude that the ribonucleic acid of the cytoplasm is associated with proteins, which, upon denaturation, are rich in —SH groups. Thus, they are *sulfhydryl ribonucleoproteins*. Figure 89 (1, 2) shows the localization of these substances when one uses the nitroprusside reaction on the entire egg. It will be recalled that the germinal vesicle of the oöcyte at the end of its growth period is characterized by a high content of —SH groups, and that during maturation these groups move with the

nuclear sap to the animal pole. During the first few hours following fertilization the sulfhydryl proteins tend to move to the equator of the egg, where they form a crescent or a ring enlarged on one side. Now, there is reason to believe that this side is the dorsal side but the proof is as yet lacking. If this is really true, the nuclear sap, then, tends to accumulate in the dorsal half of the egg, chiefly in the "grey crescent," which is the first sign of the organizer.

During cleavage, a continuous synthesis of sulfhydryl nucleoproteins takes place, occurring chiefly in the animal half and at this stage a demonstrable animal-vegetal gradient is observed. The nucleoproteins are found mostly in the periphery of the cells, in the

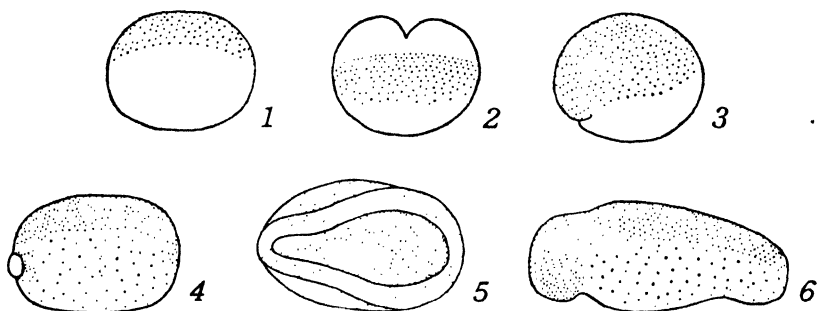


Fig. 89. Localization of sulfhydryl proteins during development in amphibia (J. Brachet). 1, fertilized egg; 2, beginning of first cleavage; 3, young gastrula; 4, end of gastrulation; 5, young neurula; 6, late neurula.

neighborhood of the membranes, and they also form a part of the asters and the spindle. Thus we see that the anabolites of Kedrowski can be detected at the beginning of cleavage and that they show a definite distribution.

At gastrulation (Fig. 89—3, 4) the nitroprusside reaction is, in the majority of cases, more intense in the region of the dorsal lip of the blastopore. It is present up to the animal pole from which it decreases rapidly toward the ventral side. At the end of gastrulation, the blastopore is very deeply stained, and a second concentration of stain is often found at the point where the anterior end of the medullary plate makes its appearance. As seen in Figure 90\* the

\* The unpublished photomicrographs of our own embryos reproduced in the plate were kindly taken by Dr. Chèvremont to whom we extend our most hearty thanks. Figures 90 and 91 are on Plate I.

ribonucleoproteins follow parallel changes. The same gradients are found, but it appears that during invagination a decrease in the content of ribonucleic acid takes place. As can be seen in Figure 91, this acid accumulates at the interstices separating the neural plate from the roof of the archenteron; it is exactly at this place and time that the inductive processes are at work. At the same time, we note a gradient decreasing cephalocaudally in the nervous system.

At neurulation, the neural plate is characterized by an abundance of sulfhydryl ribonucleoproteins (Fig. 89—5), and the cephalocaudal gradient remains very apparent. In the chordamesoderm, the notochord and the somites contain large amounts of ribonucleoproteins, but these substances tend to disappear in regions more ventrally located. Thus at this stage we see two distinct gradients,

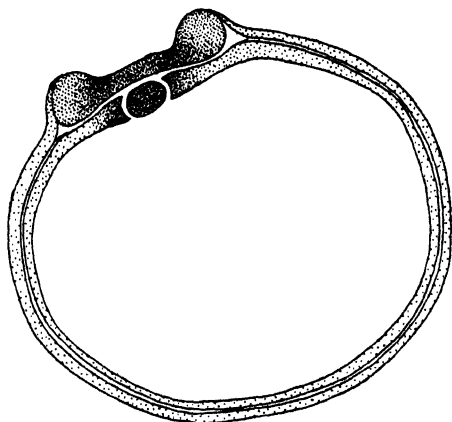


Fig. 92. Distribution of ribonucleoproteins in early neurula (diagrammatic) (J. Brachet).

cephalocaudal and dorsoventral. The lateral and ventral mesoderm, as well as the mass of endoderm, remain very low in nucleoprotein content. Figures 92 and 93 show a general view as well as a detailed representation of the distribution of these substances.

In later stages we confirmed the facts described by Kedrowski. The amount of ribonucleoprotein increases and this synthesis coincides with histological differentiation. When the latter is complete, for example in the vacuolized notochord, the concentration of nucleoproteins decreases. It will be recalled that these findings are confirmed by quantitative determinations of ribonucleic acid, which increases 60 to 70 per cent from fertilization to hatching. At gastrulation, we have observed that the dorsal half contains about 20 per cent

more pentoses than the ventral half, while in the neurula, the medullary plate and roof of archenteron are almost twice as rich in pentoses as the ventral mass. Finally, in this same stage, the anterior half of the embryo contains more ribonucleic acid than the posterior region. In view of these results, the information gathered from cytochemical studies attains its full significance.

We will not stop to consider the studies on the fish or the chick in detail, since these results complement those which we have just outlined, with the reservation that the embryo, which in these cases is separated from the yolk, alone contains the nucleoproteins. We

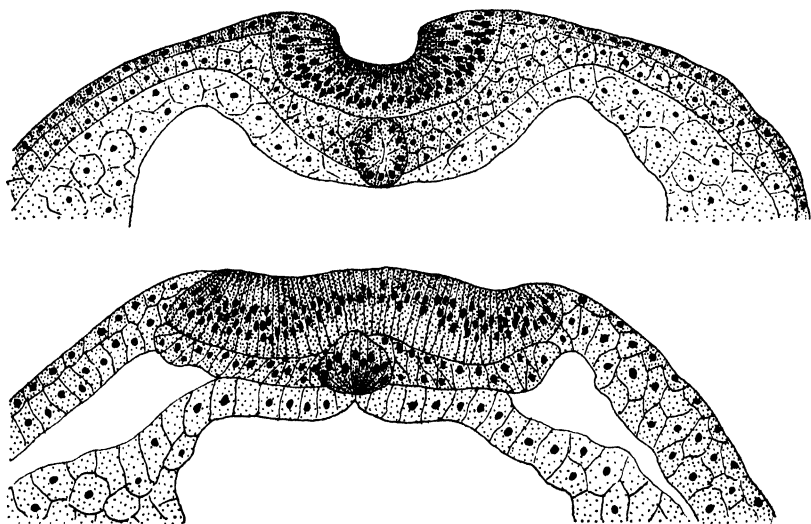


Fig. 93. Detail of distribution of the ribonucleoproteins in two stages of neurulation (J. Brachet).

find again the primary animal-vegetal gradient of ribonucleoproteins and the accumulation of these substances in the blastoporal region, while later on the dorsoventral and cephalocaudal gradients are easily distinguished. However, these facts are less demonstrable in these forms than in amphibians, except for the lamprey, which behaves exactly like the latter. The reason for the slight differences seen must be sought in the meroblastic type of cleavage which isolates the embryo from the yolk. We should mention here that the existence of a cephalocaudal gradient of the —SH groups had been reported earlier by Rulon in the chick embryo of seven to nine somites.

Our results on the distribution of the sulfhydryl proteins in amphibians have been confirmed in the main by Giolitti (1939). The Italian author, however, finds that the localizations which we have described fail to appear when the technique is modified. This should not be surprising, since the characteristic localizations are visible only when the yolk does not give the nitroprusside reaction as well. If, due to some change in methods, the yolk gives the free —SH groups reaction, then one obtains a homogenous coloration; this phenomenon actually occurs in certain amphibians even with the usual technique, because their yolk gives a positive nitroprusside test.

The parallel between the results furnished by the two methods used by Brachet leaves no doubt regarding the reality of a synthesis of sulfhydryl nucleoproteins and their localization. His observations fit in perfectly with those of Ivanov on yolk utilization, and this agreement is, indeed, inevitable since the sulfhydryl nucleoproteins can only derive their origin from the yolk which contains, as we have already pointed out, sulfur and phosphorus in quantity (Fauré-Fremiet and Dragoiu).

What conclusions may be drawn from all of these facts? The presence of an animal-vegetal gradient, the reciprocal of the yolk gradient is clearly demonstrated. At gastrulation, a new center of synthesis of ribonucleoproteins makes its appearance, localized in the region of the organizer. This is in agreement with Child's idea that the organizer is a second center of high physiological activity, appearing at gastrulation. Also worthy of note is the demonstration of the cephalocaudal and dorsoventral gradients, and it may especially be emphasized that these gradients concern the distribution of a definite substance, a sulfhydryl ribonucleoprotein. We shall see later that they also coincide with gradients of respiratory metabolism, conforming again with Child's theory. The essential result is that the ribonucleoproteins accumulate at all the places where morphogenesis is actively going on, and we now know that the nucleoproteins play a role, direct or indirect, in the synthesis of proteins. From this we may deduce that protein syntheses are especially intense in the regions which experiment has shown to be important in development. This conclusion resembles that of Kedrowski, who claimed that the anabolites participated in syntheses. However, this participation occurs at a much earlier stage than this author believed.

Among the recent studies on the cytochemistry of amphibian eggs those of Brachet and of Moyson on the localization of alkaline phosphatase may be noted, although they may not be important contributions to the problem of primary morphogenesis, scarcely doing more than extending the results obtained by Moog on the chick to the amphibians. The alkaline phosphatase reaction is weak in the cytoplasm as well as in the nucleus during cleavage and the chromosomes show no more reaction than does the chromatin of the resting nuclei. Thus it does not appear that this enzyme plays an important role in the synthesis of thymonucleic acid during cleavage. The intensity of the reaction increases a little during gastrulation and neurulation without any distinct differences between various regions of the egg. However, at the end of neurulation, organs undergoing differentiation show a more intense reaction, especially in the region of the nuclei. It thus appears clear that, in agreement with the conclusions of Moog, alkaline phosphatase takes part in the differentiation of organs rather than in primary morphogenesis. Perhaps this enzyme plays a role in the synthesis of specific proteins during their formation in different organs, Bradley and Jeener both having drawn attention to the possible coupling between alkaline phosphatase and the synthesis of fibrous proteins.

Let us conclude this section by an examination of two studies which form the bridge between cytochemical studies and quantitative observations on respiratory metabolism. We refer to the studies on the *anaerobic reduction of vital dyes in Triton cristatus* by Fischer and Hartwig, who have investigated the reducing power of the neurula, and by Piepho, who concerned himself with earlier stages, notably the gastrula. The technique applied by Fischer and Hartwig consisted in placing the embryo, from which the jelly had been removed, in a dye, generally brilliant cresyl blue, which had previously been reduced by hydrogenation. At the end of a certain time, the egg is re-exposed to air and the leucobase is re-oxidized, and in this way the embryos become rapidly colored in a very regular manner. Then they are placed in a special chamber filled with pure nitrogen where the rate of reduction of the dye in various regions may be followed under a binocular.

Fischer and Hartwig obtained the expected picture in the neurula, since it was always the neural plate and later the neural groove which first reduced the dye, the decoloration often beginning at the anterior end. The medullary folds did not differ from the rest of

the epidermis in reducing power. In later stages, Fischer and Hartwig noted a more rapid reduction in the region of the tail bud, the eye, the auditory vesicle, and the gills. They cautiously concluded that an increase in reducing power does not imply an increased respiration, but is merely an index of intensified intermediate oxidation-reduction. In any event, it is established that neural cells have the most active reducing systems (dehydrogenases?) at the time of neurulation.

Using the same technique, Piepho also arrived at some very interesting conclusions. In the blastula and young gastrula, the animal half becomes decolorized faster than the vegetal half. In middle and late gastrulae (Fig. 94) the organizer shows a higher

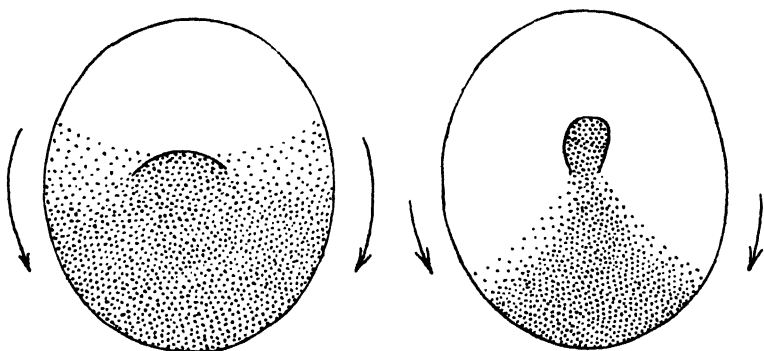


Fig. 94. Reduction gradient in early and late gastrulae (Piepho).

reducing power than the surrounding regions in 60 per cent of the cases, the dorsal lip reducing the dye almost twice as fast as the ventrocaudal region. Incidentally, Piepho noted that when the egg is injured by a needle, the injured region becomes decolorized more rapidly. This is a valuable observation since it shows that the measurements of  $rH_2$  by Dorfman using a microelectrode inserted into the egg may be subject to errors. Brachet, too, has observed that the nitroprusside reaction becomes more intense in regions which have been subjected to injuries during removal of the jelly.

Piepho also carried out investigations on the medullary plate explanted along with the roof of the archenteron of the young neurula. The nervous system alone is endowed with an intense reducing power, the underlying material, responsible for induction, not being especially active. Piepho concludes by noting that his results



fall in line with those Bellamy and Child (see Fig. 75) gathered in the course of their studies on differential susceptibility.

The results of Fischer and Hartwig and of Piepho have been confirmed by Child in another species, *Triturus rivularis*, where he recognized in the blastula the presence of a reduction gradient decreasing from animal to vegetal pole and with a greater intensity on one side (dorsal). At the gastrula stage the dorsal lip of the blastopore becomes decolorized first, the zone of reduction then extending to the animal pole. The contrast between the dorsal lip and the ventral marginal zone is best shown when one examines the gastrula from its ventral pole, as in the figures of Piepho (see Fig. 94). In the more advanced gastrula, it is the region at the periphery of the blastopore and also the anterior region of the future neural plate where reduction is most rapid. Finally in the neurula, the nervous system becomes decolorized first, following an antero-posterior gradient. Similar results have been obtained by Child in two species of fish, *Oryzias* and *Brachydanio*.

The findings of the German authors on reducing power are in perfect agreement with those of Brachet, since it is the region richest in sulfhydryl ribonucleoproteins that is decolorized first. The parallel is so close that one is tempted to think, at first, that the —SH groups reduce the dye, but such a conclusion is premature since the —SH groups were determined after fixation, and there is nothing to prove that the *living* organizer is richer in —SH groups than other regions of the egg. The nature of the reducing systems of the organizer and of the medullary plate certainly deserves a serious study, and it is only when this study is undertaken that we will be able to solve the problem.

We conclude from all these considerations that the experiments on reducing power, like the cytochemical researches on the nucleoproteins, are favorable to Child's theory, for very different procedures reveal a primary animal-vegetal gradient and confirm the later appearance of a center of activity in the region of the dorsal lip of the blastopore. The time has now come to see if these gradients are really metabolic in nature.

### 3. Metabolism of the Organizer

Studies on the metabolism of the organizer have multiplied during recent years, and we believe it is valuable to make a searching

analysis of them at this time because the investigators have often arrived at opposing points of view. This is a result of the fact that certain sources of error have only lately come to light and also because the experimental conditions used were not always the same. But when we take the trouble to go into the details of the problem, we see that the disagreements are more apparent than real and that it is possible to distinguish the most essential facts and to draw definite conclusions.

Brachet tried as early as 1934-1935 to measure the gaseous exchange of the organizer, but at this time the imperfections of technique were such that the results of these first attempts appear today to leave something to be desired. Lacking appropriate techniques, he was obliged to carry out measurements on gastrulae in which the organizer or some equivalent ventral region was destroyed by pricking (Fig. 95). The results of these lesions are very variable. De-

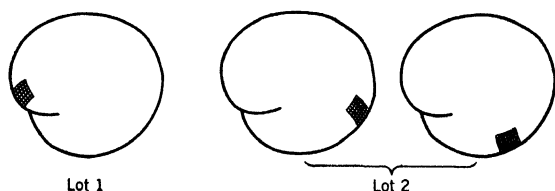


Fig. 95. Regions destroyed by puncture before measuring metabolism (J. Brachet).

velopment, after ventral puncture, was generally fairly normal, but after destruction of the organizer, a wide variety of malformations was obtained, ranging from a marked deficiency of the head region to almost complete regulation. Since the puncture itself may interfere with gaseous exchange, an interval of several hours was necessary before beginning the measurements. Further, it was very difficult to destroy exactly comparable amounts of the various regions. In a few cases the destruction of the organizer was carried out more exactly by extirpation with a platinum loop, but the closing of the wound required considerable time and thus development had gone beyond the gastrula stage when the gaseous exchange was measured. Under these conditions the metabolism of defective embryos was compared with that of almost normal partners. The result of these attempts was as follows. Respiration of embryos in which the axial organs present gross abnormalities is clearly lower than that of neu-

ruiae in which the structure is normal in spite of the operations. Thus the nervous system and the notochord have, at the time when they differentiate, a higher metabolism than ventral regions.

In seven experiments carried out on the egg of *Discoglossus* the elimination of  $\text{CO}_2$  by the isolated organizer was compared with the metabolism of corresponding ventral fragments (ventral marginal zone: Fig. 96) using a colorimetric method. In all cases a *clear difference in favor of the organizer* was found, attaining a value of 85 per cent on the average. These figures call for some comment. Development is extremely rapid in *Discoglossus*, as a result of which the isolated fragments undergo appreciable differentiation in the course of the twelve-hour experiment. This differentiation, however,

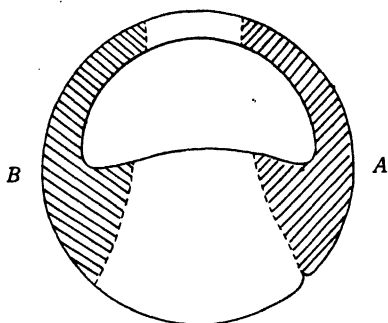


Fig. 96. Preparation of explants for measurements of metabolism. A, organizer; B, ventral marginal zone (J. Brachet).

is very unequal for the two fragments, since the organizer forms notochord and nervous system, while the ventral marginal zone remains relatively unchanged. The experiment shows that the organizer at the time when it exerts its effects possesses a powerful metabolism. Nothing, however, is proved regarding its condition in the young gastrula. It must be added, too, that the dorsal and ventral fragments were not weighed, their sizes simply being estimated by inspection. Finally, these measurements concerned only the  $\text{CO}_2$  output, the question of oxygen consumption remaining unanswered.

These first experiments did not show that the organizer of the early gastrula differed from the ventral marginal zone in the rate of oxidations but only that the axial organs, during their formation, eliminated more  $\text{CO}_2$  than ventral fragments, which are inert from the point of view of morphogenesis. It must be concluded that the increase in gaseous exchange during gastrulation and neurulation are caused, in general, by the formation of the nervous system and the

notochord. The idea that the differentiation of the embryo requires energy thus receives new support from these experiments.

Somewhat later the problem was taken up by Waddington, J. Needham and Brachet. This time it was the *oxygen consumption* of the dorsal and ventral explants which was determined by the method of Gerard and Hartline, somewhat modified (see Chapter I). The first series of experiments carried out on the gastrula of *Rana temporaria* indicated a difference of 25 per cent in favor of the organizer, but it was soon evident that this difference was not real, since it disappeared if care was taken to weigh the fragments. Thus a systematic error was introduced in the estimation of the size of the explants. Twelve measurements were carried out on the gastrula of *Triton alpestris* after taking into account this source of error, and the difference in favor of the organizer then fell to 7 per cent, i.e., within the limits of experimental error. These experiments differed from the preceding ones in several respects. For one thing, the duration was reduced so much that the development of the dorsal fragments became negligible; for another, the determination of the dry weight also gave greater assurance; and finally, the measurements dealt with the absorption of oxygen rather than elimination of CO<sub>2</sub>. The natural conclusion of this work was as follows: *the organizer does not have a higher respiration than other regions of the gastrula, but its respiratory quotient may be different*. Such a hypothesis made possible an explanation of the disagreement between Brachet's first observations and those made at Cambridge. The CO<sub>2</sub> production of the organizer is about 40 to 50 per cent greater than that of the ventral marginal zone, even if one corrects the values of 1935 by taking into account the error made in the evaluation of the size of the explants, but the oxygen consumption of the organizer amounts to only 5 or 10 per cent more than that of the ventral fragments. Thus one could predict that the dorsal lip of the blastopore had a respiratory quotient of 1 while that of the ventral marginal zone would be much lower. This hypothesis becomes the more plausible when it is remembered that the R.Q. of the entire egg changes from 0.65 to 1 at gastrulation, as we have seen in the preceding chapter.

There are other factors which must be taken into account. First, the unequal duration of the experiments and then the fact that the fragments were not taken from exactly the same region in both series of experiments. Figure 97 shows the condition of the

explants before the oxygen consumption measurements. If one compares it with Figure 96, it is seen that the ventral fragment D was purely epidermal, that is to say, it contained less yolk than the organizer C. For this reason the ventral marginal zone was selected in the earlier experiments, for, if the yolk is inert from the point of view of oxidation, we would expect that the pure epidermis of Figure 97 would have a higher respiration than the ventral marginal zone removed from the location shown in Figure 96. The result would be a smaller difference in the 1936 experiments as compared with the earlier ones.

Faced with so many uncertainties, Brachet took up the question once more in 1936 and 1939. The explants were again prepared according to the diagram in Figure 96, the amount of yolk in the dorsal and ventral fragments thus being as equal as possible under

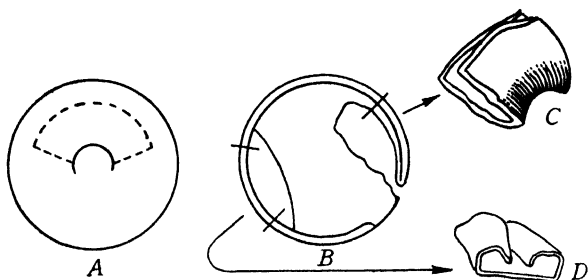


Fig. 97. Preparation of explants for measurement of metabolism (J. Needham, Waddington, etc.).

these conditions. A first series of determinations concerned the  $\text{CO}_2$  production measured by a microtitration technique, the amount of nitrogen of the explant serving as a reference in this experiment. The 1934 results were completely confirmed, an average difference of 85 per cent in favor of the organizer (for the same amount of nitrogen) being found.

In addition, the *respiratory quotient* was measured directly by preparing a large enough number of fragments so that the method of Meyerhof and Schmitt, using Warburg manometers, could be employed. The oxygen consumption of the two types of fragments differed by 31 per cent while the difference in  $\text{CO}_2$  reached 84 per cent. The results give a *respiratory quotient* of 1.02 for the organizer and 0.73 for the ventral marginal zone (average of seven

measurements). The hypothesis formulated by Waddington, Needham and Brachet thus is confirmed: a high respiratory quotient, an indication of carbohydrate metabolism, characterizes the dorsal lip of the blastopore, which is where the oxidation of sugars begins during gastrulation.

Concerning the 30 per cent higher oxygen consumption of the organizer, it is important to note that the experiment was carried out on *Discoglossus* and lasted from 20 to 30 hours. Very probably the dorsal explants underwent considerable differentiation during this time, while no morphogenesis went on in the ventral fragments. These measurements therefore do not permit us to draw any new conclusions regarding the oxygen consumption of the organizer in the young gastrula.

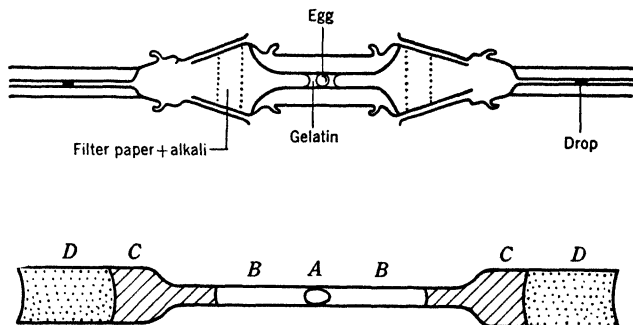


Fig. 98. Apparatus used by Brachet and Shapiro for determination of oxygen consumption (*upper*) and the production of  $\text{CO}_2$  (*lower*) of the intact gastrula. Explanation in text.

Measurements carried out on explants are subject to an objection, for the fate of isolated areas is modified and new developmental potencies are expressed. The results on the metabolism of fragments can therefore not be transposed directly to the intact gastrula. In order to avoid this difficulty, Brachet and H. Shapiro adopted a new technique (Fig. 98, top). It consisted in placing a gastrula in a glass capillary and then orienting it in such a way that the blastopore faced one end of the tube, a fine layer of gelatin fixing the egg in position without interfering with the diffusion of oxygen. A second, much finer capillary connects with the first and the displacement of a small drop of kerosene in the second capillary is observed under

the microscope. In the joint which serves to connect the two capillaries a roll of paper moistened with NaOH solution is placed for the absorption of the  $\text{CO}_2$  produced by the egg. In this way, each half of the intact egg respire by itself and the displacement of the drop of kerosene corresponds to the absorption of oxygen.

Numerous determinations were made on the gastrula of *Rana sylvatica*, leading to the conclusion that *the dorsal half has a respiration 47 per cent higher* than that of the ventral hemisphere. When the orientation of the embryo in the capillary is such that right and left halves are measured, there is no detectable difference in metabolism, thus providing a valuable control.

Brachet (1939) re-examined the problem in *Rana fusca* with an improved technique which reduced the duration of the experiment and lowered the temperature of the bath. This time only a difference of 7 per cent in favor of the dorsal half was found, and, in addition, in two out of eleven experiments the ventral hemisphere showed the higher respiration.

At the same time the  $\text{CO}_2$  production was measured using the apparatus in Figure 98 (lower). The egg is introduced into a capillary A, and on each side of it spaces of equal size, B and C, are filled with a definite quantity of a solution containing a known amount of bicarbonate and some phenol red. The tube is sealed at each end with oil, D, and then shaken in a constant temperature bath. At regular intervals the pH of the liquid C is measured by a colorimetric method and one calculates the amount of  $\text{CO}_2$  produced. Control experiments have shown that ammonia production, which would falsify the results, is very low and, furthermore, is the same for the two halves of the egg.

These measurements, numbering forty-three, have shown without doubt that the  $\text{CO}_2$  production is higher in the dorsal half, the average difference being 23 per cent. Here again control experiments, in which the metabolism of the right and left halves is compared show no appreciable difference. If the respiratory quotients of the dorsal and ventral halves be calculated, we find 0.96 and 0.80, respectively. These values agree satisfactorily with those found with explants by an altogether different procedure. One may then conclude that *in the intact gastrula the organizer has a respiratory quotient of about 1, while the ventral half is characterized by a much lower coefficient.*

## **PLATES I-VII**

**Figures 90, 91, 104, 105, 106, 107, 109,  
110, 111, 112, 113, 115, 117, and 118**



# PLATE I

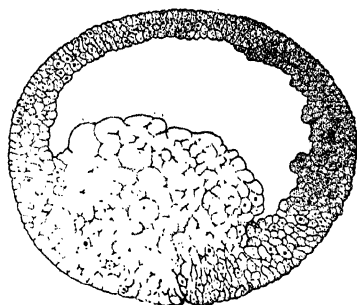


Fig. 90a. Gradient distribution of ribonucleic acid in the young gastrula. See page 358.

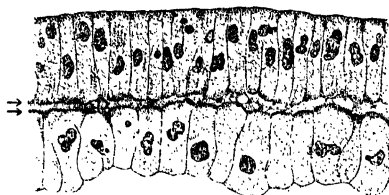


Fig. 90b. Gradient distribution of ribonucleic acid in the advanced gastrula. See page 358.

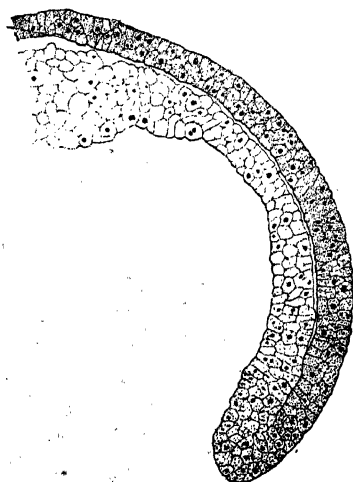


Fig. 91. Accumulation of ribonucleic acid at the interstice between the neuroblast and the roof of the archenteron at the end of gastrulation. See page 359.

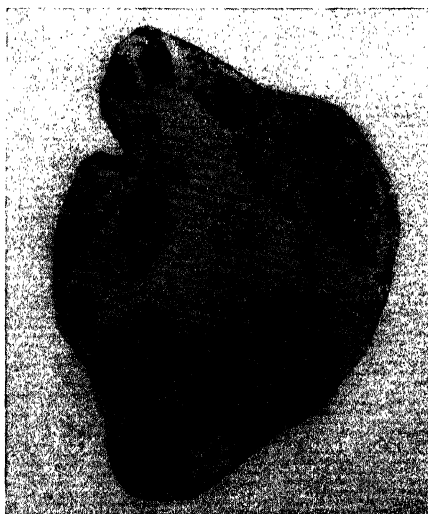


Fig. 104. Induction of a secondary neural tube (*bottom*) by the implantation of a fragment of epiblast treated with methylene blue; this fragment has also undergone a neural differentiation (Waddington, J. Needham, and Brachet). See page 387.



Fig. 105. Differentiation of nervous system in a dorsal fragment of a gastrula treated with cyanide. See page 389.

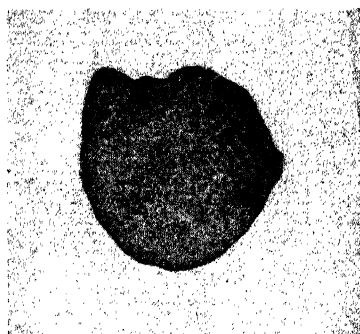


Fig. 106a. Dorsal explant treated with oxidized glutathione; the nervous system (*top*) retains the palisade-like cell arrangement. See page 391.



Fig. 106b. Control dorsal explant. See page 391.

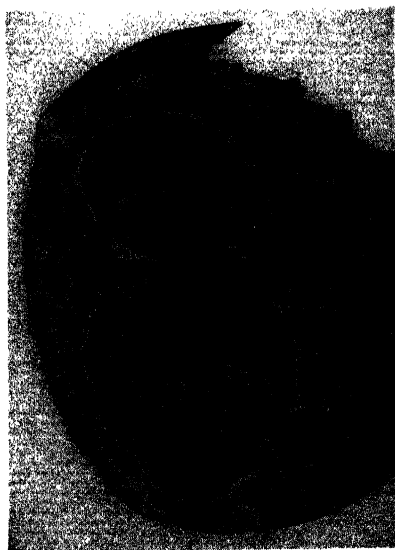


Fig. 106c. Differentiation of nervous system and notochord in a ventral explant treated with cysteine. See page 391.

# PLATE III



Fig. 107. Differentiation of a neural mass in a ventral explant treated with sulfocyanide (Ranzi and Tamini). See pages 391 and 392.

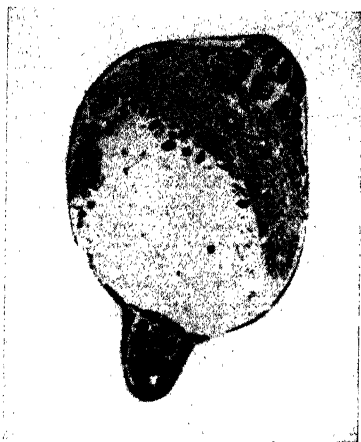


Fig. 109a. Induction of a secondary neural tube (*bottom*) after implantation of a coagulated extract of neurulae (J. Needham, Waddington, and D. Needham). See page 399.



Fig. 109b. Neuroid reaction (*bottom*) after grafting a petroleum ether extract of neurulae (J. Needham, Waddington, and D. Needham). See page 399.



Fig. 109c. Slightly palisade-like reaction (*top*) after implantation of the unsaponifiable material from neurulae (J. Needham, Waddington, and D. Needham). See page 399.



Fig. 110a. Good neural induction (*bottom*) after implantation of an ether extract of glycogen (Waddington, J. Needham, Nowinski, and Lemberg). See page 400.



Fig. 110b. Neuroid reaction (*bottom*) after implantation of the unsaponifiable material from liver (Waddington, J. Needham, Nowinski, and Lemberg). See page 400.



Fig. 111. Neuroid induction after grafting a synthetic polycyclic hydrocarbon (Waddington and D. Needham). See page 400.



Fig. 112. Good neural induction obtained by the implantation of a dead organizer from which the fat had been completely removed (Holtfreter). See page 401.

## PLATE V



Fig. 113a. Induction of a medullary plate after implanting synthetic oleic acid (Fischer). See page 401.

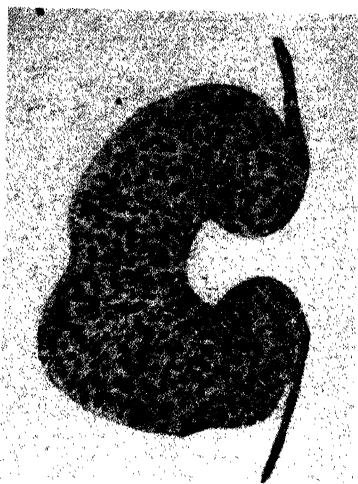


Fig. 113b. Induction of a medullary plate after implantation of a thymonucleic acid (Fischer). See page 401.

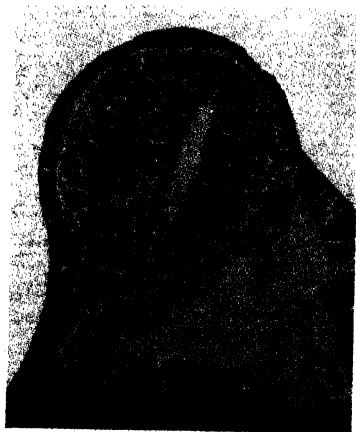


Fig. 115. Good neural induction after implantation of a polycyclic hydrocarbon (derived from sterols) (Waddington). See page 405.



Fig. 117a. Control frog embryo. See page 412.



Fig. 117b. Frog embryo treated with iodoacetamide; the nervous system remains compact and palisade-like. See page 412.

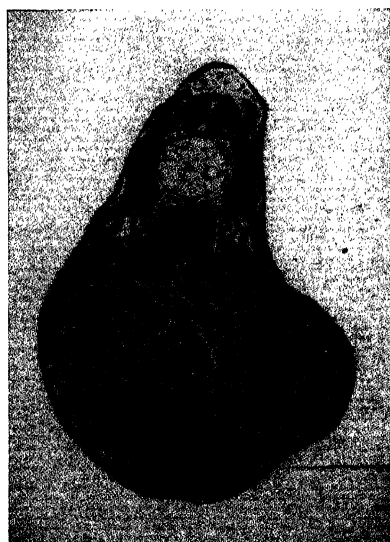


Fig. 117c. Control frog embryo (at a more posterior level). See page 412.

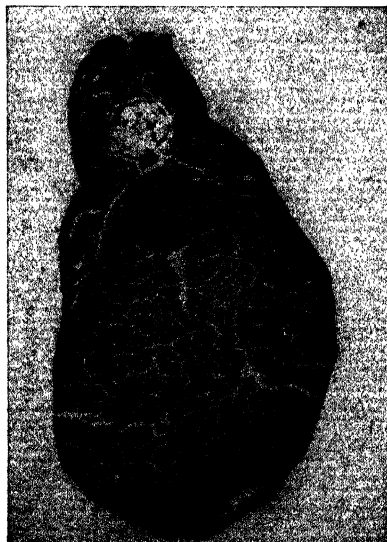


Fig. 117d. Embryo treated with iodoacetamide; the nervous system is reduced to an undifferentiated mass. See page 412.

# PLATE VII

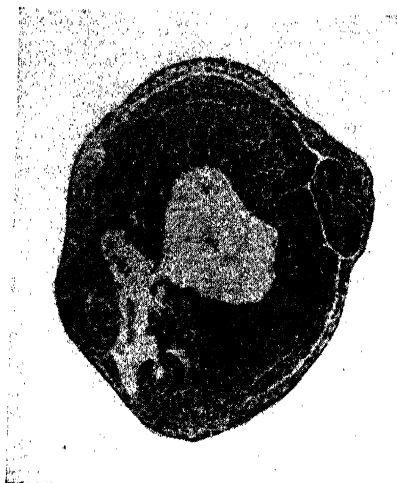


Fig. 118a. Induction of a secondary neural tube (*lower left*) by grafting nucleoproteins (granules) of liver. See page 415.

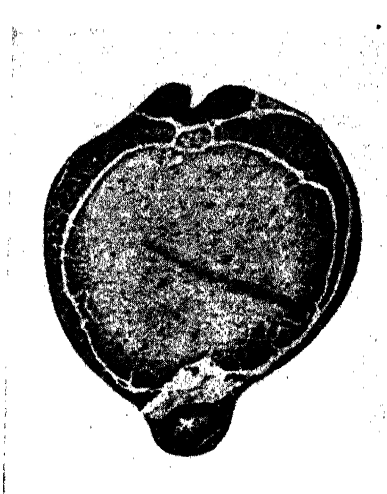


Fig. 118b. Induction of a secondary neural tube (*bottom*) by grafting nucleoproteins (granules) of kidney. See page 415.

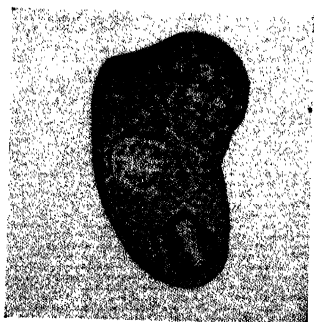


Fig. 118c. Induction of a neuroid mass (*upper right*) after implantation of nucleoproteins (granules) of yeast. See page 415.

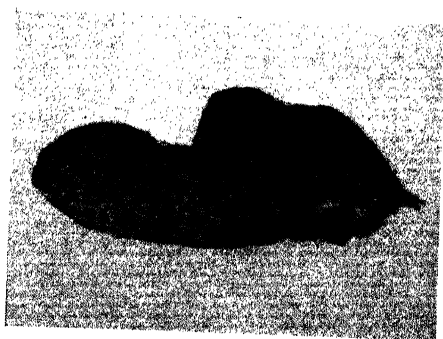


Fig. 118d. Induction of medullary plate after implantation of crystallized tobacco mosaic virus. See page 415.

We have yet to explain the different figures obtained for oxygen consumption in *Rana sylvatica* and *Rana fusca*. The fact that we are dealing with different species may be involved, but this does not appear to be the essential factor. We must then assign this discrepancy to certain of the experimental conditions. In *Rana sylvatica*, where the experiments lasted six hours at 26° C., gastrulation progressed considerably during the measurements, and the chordamesoderm invaginated completely, with the result that the thickness of the layer on the dorsal side doubled. Actually, if we take into account only the measurements of the first two hours, the difference between the two halves drops from 46 to 23 per cent in *Rana sylvatica*. It must be admitted, as noted by Fischer and Hartwig, as well as by Boell and J. Needham, that the amount of actively respiring material increases on the dorsal side during gastrulation; it would, however, be valuable to bring further proof by planimeter measurements carried out on gastrulae placed in capillaries and fixed in various stages in gastrulation.

The interpretation of the results in *Rana fusca* is certainly much simpler. The oxygen consumption of the dorsal half is practically identical at the young gastrula stage with that of the ventral hemisphere, but its respiratory quotient is higher. This conclusion leads us to suppose that carbohydrate metabolism begins in the dorsal lip of the blastopore. It seems clear that the respiration of the dorsal region increases more rapidly during gastrulation than that of the ventral region and it is probable that at the end of invagination, that is to say, when the organizer fully exerts its influence as an inductor, respiration of the dorsal half is higher. *The difference between the dorsal lip and the ventral marginal zone is thus at first qualitative and does not become quantitative until the time when gastrulation is completed.* We will see that some observations agreeing with this conclusion have been made by other investigators.

The question of the metabolism of the organizer has recently stimulated a number of investigations. The problem has been studied by Needham, Boell and their collaborators in England, Fischer and Hartwig in Germany, Barth in the United States, and Stefanelli in Italy. A discussion of the significance of their findings seems to be in order here.

Fischer and Hartwig have measured in *Triton* the oxygen consumption of the isolated dorsal lip and compared it with the pre-



sumptive ventral epidermis, the latter being taken from between the ventral marginal zone and the animal pole and therefore containing less yolk than the organizer. The measurements, carried out with Warburg manometers of small volume, were made on a large number of explants (20 to 60). The dry weight of these explants was determined at the end of the experiment. A series of twenty-seven experiments led Fischer and Hartwig to the conclusion that the respiration of the organizer is about 20 per cent greater than that of the presumptive epidermis. In studying more closely the protocols of Fischer and Hartwig, however, it is seen that the difference in favor of the organizer reached 29 per cent when the measurements extended over sixteen to twenty-three hours, the difference dropping to 11 per cent when the duration of the experiment did not exceed six to eight hours. These findings agree perfectly with Brachet's since he found a 31 per cent greater respiration in experiments on explants of long duration and only 7 per cent in measurements of brief duration on the entire egg. Fischer and Hartwig concluded that the organizer has a higher respiration than the ventral regions, but it appears more cautious to say that its metabolism is only slightly higher than that of presumptive epidermis at the beginning of gastrulation and that oxidation in the dorsal explants rises more rapidly during development than the ventral fragments.

Similar results were obtained by H. Lehmann, a pupil of F. G. Fischer. Two of his graphs of respiration are reproduced here (Fig. 99), and it may be clearly seen that the difference between the two curves becomes greater with time.

In extending their measurements to other stages of development, Fischer and Hartwig have established mainly that oxidation in the *animal half of the blastula* is more than three times that of the vegetal half—the figures are 1.67 and 0.53 mm<sup>3</sup> of oxygen per 10 mg per hour, respectively. It must be concluded, then, that the yolk does not participate directly in oxidation, behaving in this respect as an inert mass.

At the *neurula* stage, the *medullary* plate shows a respiration of 2.6 times the entire larva, thus constituting a center of intense oxidation. This agrees very well with the idea stated earlier that the increase in oxidation during gastrulation and neurulation is due, in the main, to the differentiation of axial organs. Finally, for advanced neurulae Fischer and Hartwig have established that the

cephalic end consumes more oxygen than the caudal half (in the proportion of 2.7 to 1).

Stefanelli also arrived at analogous results (1938, 1939), working with frog and toad embryos and using a microrespirometer of his own design. Unfortunately, he did not think it necessary to determine the weight or nitrogen content of the fragments and relied on subjective estimations of the equality of the size of the fragments. We have already seen that this procedure is subject to errors and

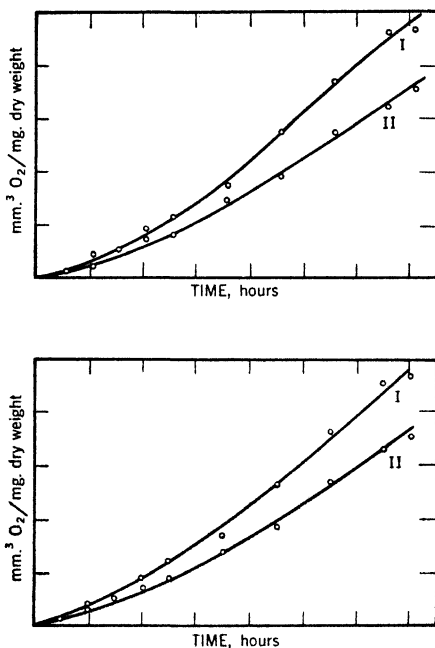


Fig. 99. Oxygen consumption of the organizer (I) and ventral epidermis (II) (H. Lehmann).

the figures given by Stefanelli are only approximate from a quantitative point of view. He was not able to detect differences between dorsal and ventral explants of the young gastrula, since it was not until just before the thickening of the medullary plate and especially at the time of its appearance that he saw a clear increase in the metabolism of the dorsal regions. At neurulation, a cephalocaudal gradient in the nervous system was observed but it involved small

variations of the order of 5 per cent, whose existence is especially doubtful since neither the weight nor total nitrogen of the explants was determined. The caudal extremity of the nervous system was seen to absorb more oxygen than the neighboring epidermis, the difference attaining 25 per cent. The results of Stefanelli thus confirm the idea that oxidation in the organizer is not particularly intense at the beginning of gastrulation, it being only later, at the time of induction proper when the nervous system arises, that the metabolism of the dorsal organs becomes very high. Stefanelli concludes from this that it is necessary to reject Child's theory since the organizer does not have a high metabolism at the time when it is already an inductor. We are of the opinion, however, that the inductive power of the early dorsal lip has not really been demonstrated, since when one transplants this region into a host the fragment continues to differentiate, and perhaps does not induce until the time when it is completely invaginated. In any case, we have not obtained inductions by grafting dorsal lips of the blastopore isolated from gastrulae whose development has been blocked by various means. Thus it seems that the young dorsal lip is only a poor inductor, and the fact that its metabolism is not markedly higher until the time of induction is thus not contradictory to Child's theory, which couples induction with increased respiration.

The beautiful work of Needham, Boell and their collaborators on the metabolism of the organizer will concern us at length, for it is noteworthy for the care in manipulation and the control of the technique used for the determinations. However, their interest lies chiefly in the fact that the analyses were not limited to oxygen consumption, but included the *respiratory quotient* and anaerobic and aerobic *glycolysis* as well. All these measurements were made by means of the Cartesian diver of Linderstrøm-Lang, which in the hands of Needham and Boell has become an exceptionally useful instrument. Since a micromethod for the determination of nitrogen was used, analyses became possible for a single explant, thus insuring a quantitative basis for comparing the dorsal and ventral explants obtained from the same gastrula. Finally, the measurements are sufficiently numerous for a statistical analysis.

Let us examine first the results of Needham and Boell in regard to *oxygen consumption*. Explants were prepared according to the diagrams in Figure 97. We have noted that the layer of ventral

epidermis has, under these conditions, less yolk than the organizer. For *Discoglossus*, Boell and Needham found no appreciable differences between the two types of fragments in experiments which did not exceed three hours duration. If the results are expressed as  $Q'_{O_2}$ , i.e., the oxygen consumption in cubic millimeters per milligram of nitrogen per hour, one obtains a value of 4.80 for the organizer and 4.93 for the epidermis. When the values are expressed in terms of dry weight, the figures become 0.35 and 0.33, respectively. In axolotl, the  $Q'_{O_2}$  of the dorsal lip is 3.21, that of the ventral fragment 3.18. Boell and Needham thus concluded that there is no real difference between the two types of explants.

This work calls for some comment. Although the chemical techniques adopted seem irreproachable, the biological conditions do not fulfill all the desired requirements. In the diver the explants are actually immersed in only 2 mm.<sup>3</sup> of the fluid, and the cells are thus in contact with glass and separated from air by only a thin aqueous film (Fig. 100). Now, everyone who has handled fragments of gastrula knows their fragility, so in experimental embryology one always carefully avoids contact of the cells with glass. In addition, the least contact with air causes an immediate cytolysis in the tissues of the gastrula, and we know from the preceding chapter that cytolysis of the egg profoundly modifies its metabolism. One regrets, then, that Boell and Needham made no reference to the state of their explants at the end of the experiment, and also that they do not say whether oxygen consumption remained constant during the three hours of measurement. This latter figure would be an index of a normal physiological state.

We fear all the more that this source of error was present by reason of a close examination of the protocols published by Boell and Needham, which, in several cases, show variations of the order of 1 to 5 for fragments coming from the same embryo. This cannot be a question of errors due to the instruments and it is probable that one of the explants was cytolized. Boell and Needham noted that the gastrular fragments from axolotl were more resistant than those from *Discoglossus* and this seems to confirm the suspicion that the experimental conditions from a biological point of view were not quite rigorous. It may be further noted that Boell and Needham have, in a few cases, measured the  $Q'_{O_2}$  of the nervous system at the time of its closure, finding a value of 3.0, which is much less than

for gastrular fragments (4.8–4.9). This result appears paradoxical, for the metabolism of the advanced neurula is much greater than that of the gastrula. The findings of Fischer and Hartwig, who observed for example, that the  $\dot{Q}_{O_2}$  rose from 1.9–2.2 to 3.1 when they compared gastrular fragments with the neural plate, appear much more reasonable in this regard. But these criticisms do not apply to more than a small number of experiments in which an abnormally

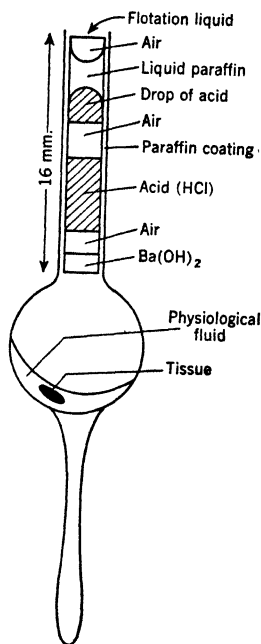


Fig. 100. Cartesian diver adapted for measurement of the respiratory quotient (Boell, Koch, and J. Needham).

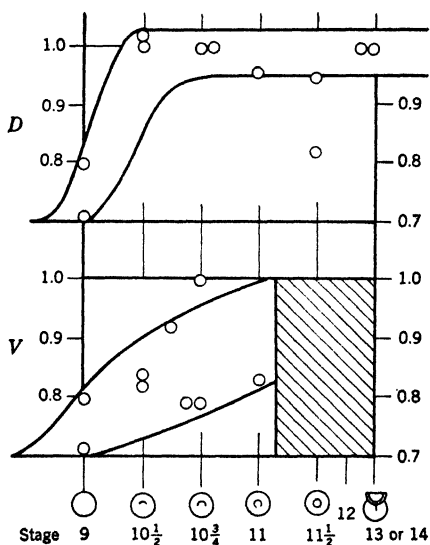


Fig. 101. Variations in the respiratory quotient in the organizer (D) and ventral epidermis (V) during gastrulation (Boell, Koch, and J. Needham).

large difference was obtained between dorsal and ventral fragments. If we disregard these, however, the evident conclusion of Boell and Needham's experiments remains that the *oxygen consumption of the organizer and that of the ventral epidermis are of the same order of magnitude* when the respiratory period measured is short and the early gastrula is used. These conclusions, furthermore, are in perfect

agreement with those which we have just seen to emerge from the work of Fischer and Hartwig, Stefanelli, and Brachet.

Boell, Koch and Needham next devised a delicate method for the measurement of the respiratory quotient of isolated fragments (Fig. 100). Using a diver with a long neck, they placed the explant in the bottom in 2 mm.<sup>3</sup> of saline solution, and into the paraffined neck of the diver was put first a drop of barium hydroxide to absorb CO<sub>2</sub>, next two drops of HCl, and then a seal of paraffin oil. The CO<sub>2</sub> formed by the explant is absorbed by the alkali and the amount of oxygen absorbed can thus be measured. At the end of the experiment the hydrochloric acid and the alkali are forced into the main chamber of the diver by a special mechanism. The medium becomes acid and the CO<sub>2</sub> combined with the alkali is then measured as well as that which is chemically combined in the saline solution or in the embryo. A similar procedure at the beginning of the experiment gives a measure of the CO<sub>2</sub> initially present. In short, they used the classical methods of Meyerhof and Schmitt and of Dickens and Simer on the scale of the Cartesian diver.

During the course of this research Boell, Koch and Needham necessarily measured the  $Q'_{O_2}$  once more, and this time they found, in axolotl, a clear difference in favor of the organizer (5.3 in comparison with 4.2 for the ventral ectoderm). The difference reduces to 3.5 and 3.2, however, if certain abnormally high values are discarded, and the authors decline to attribute any significance to this difference of 10 per cent because of the limited number of experiments. The results obtained for the respiratory quotients completely confirm Brachet's (1936, 1939) measurements. As shown in Figure 101 the R.Q. of the organizer rises rapidly to about unity (0.98), while that of the ventral epidermis is maintained at a low value (0.86) and does not reach unity until gastrulation is completed.

A few determinations on the animal half of the blastula and on the medullary plate, in axolotl, also support these conclusions, the respiratory quotient being 0.75 in the first case and 1 in the second. We have already shown that the R.Q. of the frog's egg is only 0.65 during cleavage while that of the neurula is about unity.

Finally, a series of measurements carried out on *Rana fusca* confirm once more the fact that the R.Q. of the organizer is greater than that of the ventral epidermis, the values obtained are 0.92 and 0.81, respectively.

Table II summarizes the results obtained in this field. Their agreement appears very satisfactory, considering the technical difficulties which determinations of the R.Q. present on this scale.

One can definitely state that there are qualitative differences in the metabolism of the various regions of the amphibian gastrula, differences that favor the hypothesis that the catabolism of carbohydrates first begins in the dorsal lip of the blastopore.

Before reviewing the elegant experiments which the Cambridge school has devoted to the demonstration of this hypothesis, let us finish with the question of possible *quantitative* differences between the two halves of the gastrula. We have seen that recent work agrees in recognizing that if such differences exist they cannot exceed 10 per cent. Barth is inclined toward a similar conclusion in a study which contributed largely to the clarification of a situation which remained, after all, somewhat confusing.

Table II

Species studied	Organizer	Ventral region	References
<i>Discoglossus</i>	1.02	0.73	J. Brachet (1936, 1939)
<i>Rana fusca</i>	0.96	0.80 (intact egg)	J. Brachet (1939)
<i>Rana fusca</i>	0.92	0.81	Boell, Koch and Needham (1939)
<i>Axolotl</i>	0.98	0.87	Boell, Koch and Needham (1939)

Barth isolated, in *Amblystoma* and *Rana pipiens*, a series of fragments from the gastrula, representing the dorsal lip of the blastopore (chordamesoderm), the presumptive nervous system, the presumptive epidermis, the future ventral lip (ventral mesoderm) and the vegetal pole (endoderm). The results are expressed in cubic millimeters per hour per 100 mg. dry weight and the measurements were carried out according to a modification of Winkler's technique for dissolved oxygen. Table III gives a summary of Barth's results.

Barth concluded from these data that the egg possesses an *animal-vegetal metabolic gradient*, which drops rapidly on the ventral side and much more slowly in the dorsal half. The organizer respire at a lower rate than the presumptive nervous system, at a rate very close to that of the ventral ectoderm, but its metabolism is always greater than that of the presumptive ventral lip (ventral marginal zone of Brachet's experiments). It can be seen that the results will

differ if one compares the organizer with the ventral marginal zone instead of with the ectoderm; the latter, containing less yolk, will have a distinctly higher metabolism than the future ventral lip.

The work of Barth gives for the first time a clear idea of oxidative differences in various parts of the gastrula, putting, as it does, the emphasis on the importance of the yolk content of the explants. The animal-vegetal respiratory gradient is indeed very sharp and the difference between the two poles may reach a proportion of 1 to 4 and even 1 to 7. This difference results in part from the unequal distribution of the yolk. Using two different methods, Barth arrived at the conclusion that the amount of yolk increases from 25 to 50 per cent in passing from one pole to the other. The fact that the presumptive nervous system respire more actively than the epidermis is certainly interesting, but it may be asked if this is not a

Table III

Average figures <sup>a</sup>	<i>Amblystoma punctatum</i>	<i>Rana pipiens</i>	<i>A. opacum</i>
Ectoderm	32.5	35	22.5
Dorsal lip	33.	33	13.
Ventral lip	21.	27	8.6
Endoderm	—	8	5.

<sup>a</sup> These figures are corrected for the yolk content of the various fragments.

consequence of the unequal amount of yolk in these two territories. The whole point in knowing whether the metabolism of the organizer is greater than that of the ventral epidermis loses much of its interest in the light of Barth's observations, for, according to whether the section is taken from a region a little higher or a little lower, the results will vary. What is important to remember is that the presumptive nervous system, a noninductor at this stage, has a higher rate of respiration than the organizer. Barth concludes that *the respiration of the organizer is higher than, equal to, or less than that of other regions, depending upon their yolk content.* The oxygen consumption of the dorsal lip of the blastopore is considered, when corrected for its high yolk content, as "relatively considerable."

Boell came to practically the same conclusion, also finding the animal-vegetal respiratory gradient described by Barth. The respiration of the organizer was lower in his experiments than that of



the animal pole but higher than that of the ventral marginal zone; in addition, he determined by centrifugation the amount of yolk in various regions of the gastrula. Comparing the results obtained from the two types of experiments, he came to the conclusion that there was an inverse relation between the amount of yolk and the rate of respiration. It can be seen, as Barth has pointed out, that there is a satisfactory parallel between these measurements and the results of the experiments on reduction of dyes (Fischer and Hartwig, Piepho, Child), the two methods agreeing in indicating that the animal pole and the dorsal lip of the blastopore are the two regions of the gastrula showing a high metabolism.

The observations of Barth coincide to a surprising degree with those which Brachet has made on the distribution of the ribonucleoproteins in the young gastrula. If we refer to Figure 89, we see that the parallel between the content of ribonucleoprotein of the various regions and their metabolic rate is striking. Clear differences are seen between the presumptive nervous system and the ventral ectoderm and between the blastopore lip and the ventral marginal zone; the animal-vegetal gradient is also evident. These facts should not surprise us, since we have seen in Chapter VI that ribonucleoproteins form an integral part of the granules which are the site of extremely important respiratory enzymes (cytochrome oxidase and succinic dehydrogenase chiefly).

Hence the idea arises that the intensity of respiratory metabolism in the various regions of the egg is dependent on the relative proportion of ribonucleoproteins and yolk which they contain. Without emphasizing the importance of this concept at this time it may be noted that it dovetails well into the theory of Dalcq and Pasteels. It will be recalled that these authors attributed morphogenesis to an interaction between the yolk and a substance having a distribution decreasing dorsoventrally. The organizer corresponds precisely to a region which combines a high yolk content with a high concentration of ribonucleoproteins, a condition that would also explain its "relatively considerable" respiration.

If the views just presented are correct, we may expect little or no local differences in the metabolism of eggs in which there is a homogenous distribution of ribonucleoproteins. We have already seen that in the sea urchin the respiration of animal and vegetal halves is identical (Lindahl and Holter), and recent research by

Philips predicates the same situation for the chick. Using the Cartesian diver of Linderstrøm-Lang, Philips found that the oxygen consumption of anterior and posterior halves of a two- to three-day embryo was the same. In seventeen-hour embryos, he was not able to detect differences between the head process and the anterior, middle, or posterior regions of the primitive streak, but it must be pointed out that the probable error in these experiments was very high. We have seen that gradients in ribonucleoproteins are not very evident in this case, although closer study demonstrates them. It is possible that measurements carried out on very small groups of cells taken from regions having the highest and lowest concentration of ribonucleoproteins would reveal differences in metabolism even in the chick embryo.

In spite of data from various sources which have been brought together over the course of the last few years, it does not seem yet possible to discuss Child's theory in relation to the amphibian egg. The idea that the organizer is a region of very high metabolism, predominating in this respect over all other regions of the gastrula, must be discarded. This idea is not really part of Child's concept, however. It will be recalled that he considered the organizer as a secondary center of metabolism superimposed on the primary respiratory gradient which decreases from animal to vegetal pole. Now, the reality of this gradient is no longer questioned, but we still do not know if the metabolism of the organizer becomes "relatively considerable" only at gastrulation or if it is the same in the antecedent dorsal marginal zone of the blastula. One can only pose the question and point out that experiments that would supply the answer are perfectly feasible. Child's theory appears at present as too unilateral, for certainly factors other than respiratory metabolism exercise a real influence, notably the relative proportions of ribonucleoproteins and yolk. Furthermore, there is one point in this theory that it is not possible to accept. Child thinks of these gradients as being exclusively quantitative, but there is no doubt that *qualitative* differences, revealed by measurements of respiratory quotients, also enter into the picture. We have seen that there is some evidence in favor of a carbohydrate metabolism in the organizer, and it is appropriate to discuss it now at greater length.

. In 1935 N. G. Heatley determined the glycogen content of the various regions of the gastrula for the purpose of clarifying the dis-

crepancy between the cytochemical findings of Woerdeman and of Pasteels (see p. 105). To do this he used a most sensitive method for the microdetermination of this polysaccharide. Here are the figures for the gastrula of *Triton alpestris*:

	Glycogen, % dry weight
Presumptive nervous system .....	19.7
Presumptive ventral epidermis .....	15.8
Presumptive mesoderm .....	11.5
Presumptive endoderm .....	7.0

This first series of measurements confirmed the presence of a glycogen gradient decreasing from animal toward vegetal pole, and the appreciable difference between the presumptive nervous system and ventral epidermis shows the existence of a dorsoventral gradient. Heatley interpreted the low figures for the mesoderm, which invaginates, as compared to the presumptive nervous system, which remains on the surface, as a sign of glycogenolysis during invagination, since 35 per cent of the glycogen disappeared when the organizer invaginated during gastrulation. The evidence, however, was not convincing because there was no proof that the amount of glycogen was not lower in the mesoderm than in the neural ectoderm even be-

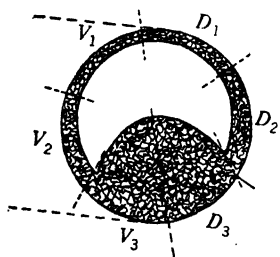


Fig. 102. Different regions of the egg analyzed for the amount of glycogen (Heatley and Lindahl).

fore invagination. The question could not be resolved until a comparison was made of the glycogen of the organizer at the beginning and at the end of gastrulation.

This was carried out by Heatley and Lindahl. The comparison was made on early and late gastrulae; Figure 102 shows the location of the extirpated regions. D<sub>1</sub> corresponds to the presumptive nervous system; D<sub>2</sub> to the organizer; V<sub>1</sub> and V<sub>2</sub> to the epidermis and D<sub>3</sub> and V<sub>3</sub> to the endoderm.

A statistical analysis shows that only the 31 per cent difference in the organizer ( $D_2$ ) is really significant, although perhaps it may also be so for the glycogenolysis in the endoderm ( $D_3$  plus  $V_3$ ).

The results of this elegant demonstration lead to a conclusion somewhat intermediate between those of Woerdeman and of Pas-teels. The organizer is, without doubt, a region of important glycogenolysis, but there is not the almost complete disappearance of glycogen as Woerdeman believed. It is also established that a *large majority of the glycogen used during gastrulation disappears during invagination of the organizer*. The latter is thus clearly an active center of carbohydrate catabolism.

Heatley and Lindahl have further shown that the glycogenolytic breakdown is chiefly that of desmoglycogen bound to the proteins, a phenomenon already ascertained by Needham and Brachet. The various regions appear to behave in the same way in this regard.

Glycogen in Per Cent Dry Weight

Fragment	Early gastrula	Late gastrula	Decrease in per cent
$D_1$	17.8	16.5	7
$D_2$	12.0	8.3	31
$V_1$	16.7	16.5	1
$V_2$	10.0	9.3	7
$D_3 + V_3$	4.3	3.9	9

The results of Heatley and Lindahl were greatly extended by L. Jaeger in an excellent investigation where the methods of experimental embryology were combined with those of microchemistry. Jaeger obtained glycogenolysis values of about 30 per cent in the dorsal lip of the blastopore, but she also reported an appreciable disappearance of this polysaccharide in the ventral lip, which is not an inductor. Further, she has found that there is no glycogenolysis in the dorsal or ventral lips when they are explanted *in vitro*, nor has she observed any loss of glycogen when the nervous system is induced after combining a fragment of ectoderm with the organizer. Hence it must be concluded that glycogenolysis is not connected in a causal manner with the inducing activity of the organizer, but rather that it serves to furnish the energy necessary for morphogenetic movements.

Another proof of the intense carbohydrate metabolism of the

organizer was found by Boell, Needham and Rogers. These investigators made use of a technique which allowed them to follow *anaerobic glycolysis* in fragments of the gastrula. After a great deal of careful effort, they succeeded in solving the delicate problem of filling the divers with nitrogen free of any traces of oxygen. The results are expressed as  $Q_L N_2$  (mm.<sup>3</sup> of CO<sub>2</sub> produced in anaerobiosis per mg. dry weight and per hour). In *Rana temporaria*, they found a value of 0.63 for the organizer against 0.21 for the ventral ectoderm. *Anaerobic glycolysis is thus shown to be three times as intense in the organizer.*

Boell, Needham and Rogers also measured ammonia production under anaerobic conditions. It shows variations according to the regions of the gastrula and the stages of development studied, as the following table illustrates:

	Mm. <sup>3</sup> × 10 <sup>-3</sup> /γ and 5 hours
Ventral ectoderm of gastrula .....	0.97
Organizer .....	2.97
Neural plate .....	2.70
Neural tube .....	1.30
Nervous system and somites of older embryos .....	0.98

Anaerobic ammonia production thus decreases after neurulation, and in the gastrula is much greater in the region of the organizer. It would be interesting to investigate the origin of this ammonia in order to see if it originates from a deamination of the aminopurines or from amino acids, for in this way one could get valuable information on the metabolism of the nucleic acids and the proteins. However, it is well to note that the formation of ammonia in the presence of oxygen is very low and that one can find no differences between dorsal and ventral regions. Perhaps the ammonia formed under anaerobic conditions serves to partially neutralize the lactic acid formed.

It may be mentioned again that in regard to the protein metabolism of the organizer the dipeptidase activity of various regions is inversely proportional to their yolk content. This result agrees well with the idea that this enzyme is localized in the hyaloplasm (G. Pickford).

Boell, Needham and Rogers have also studied the anaerobic glycolysis of explants of *Triton* embryos. The results are less satisfactory here because of the high mortality of fragments in the diver. The final figures give a  $Q_L^{N_2}$  of 0.23 for organizer and 0.14 for ventral fragments. In all the experiments, 20 in number, glycolysis was higher in the dorsal lip.

More recently, Needham, Rogers and Shen undertook to measure *aerobic glycolysis* in the two types of fragments. The results, however, were negative, the production of lactic acid being so low as to be incapable of measurement. The traces of lactic acid which the egg contains are probably neutralized by the alkaline reserve.

In any case it is established beyond doubt that the *carbohydrate catabolism* of the organizer in anaerobiosis and aerobiosis is much greater than that of ventral regions. Now that we have seen the characteristics of the metabolism of the organizer, we are prepared to examine any possible connections between metabolism and induction.

#### 4. Metabolism of the Organizer and Induction

Although the metabolism of the organizer has been studied systematically for only a short time, attempts to produce localized changes in it were undertaken much earlier. One of the most ingenious procedures consisted in placing the egg in a *thermal gradient*. If one side of the egg is heated, its metabolism increases, and a cooling of the opposite side accentuates the effect. This experiment is easily done by placing the egg between two metallic plates (thermodes) maintained at different temperatures.

J. Huxley and Vogt (1928) were the first to use this technique. Huxley heated the ventral side and cooled the dorsal half, thus applying an antagonistic thermal gradient to an assumed dorsoventral physiological gradient. He obtained microcephalic embryos as a result. Conversely, an "additive" gradient gave larvae in which head development was exaggerated: in these cases Huxley heated the dorsal side and cooled the ventral half. Tazelaar, Huxley and de Beer obtained similar results. As to Vogt's experiments, when he heated one of the first two blastomeres and cooled the other, development of the heated part was more rapid and complete.

The observations of Gilchrist have a very considerable interest from the point of view adopted in this discussion. He submitted

eggs at various stages of development to an antagonistic gradient which favored the ventral side. In addition to the usual anomalies, such as disturbances in gastrulation, asymmetries, and neural deficiencies, Gilchrist obtained in a few cases the formation of a secondary neural plate on the ventral side (Fig. 103). It would seem then that increased metabolism of the ventral hemisphere resulted in the induction of an accessory nervous system when the metabolism of the normal organizer was depressed; these facts constituted, in view of all previous evidence, valuable support for Child's theory.

The appearance of accessory nervous systems as a result of an antagonistic gradient has also been reported by Castelnovo, who has not, unfortunately, published any figures. But it is very important to know whether, in the experiments of both Gilchrist and

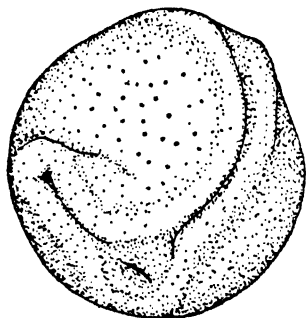


Fig. 103. *Duplicitas anterior* obtained by application of an "antagonistic" thermal gradient (Gilchrist).

Castelnovo, the secondary nervous system is fused with the primary nervous system. It may well be asked whether the thermal gradient has not simply produced some changes in gastrulation which lead to a division of the normal organizer. The anomalies pictured by Gilchrist are, as Spemann has said in his book (p. 216), duplications of the anterior parts of the nervous system. This "duplicitas anterior" can well be the result of mechanical disturbances in gastrulation which cut the roof of the archenteron in two during invagination. There is no real proof, then, that heating is sufficient to bring out inducing properties in the normally inactive ventral mesoderm.

Aware of these objections, Gilchrist (1935) repeated his experiments, using the technique of vital staining in conjunction with the thermal gradient. He recognized a "dorsal plasm" in the blastula, distributed in a dorsoventral gradient, which can be twisted and de-

formed under the influence of a temperature gradient. This explanation closely resembles the interpretation of Spemann and is rather far removed from Child's concept.

It may be added that Margen and Schechtman have more recently applied localized heating to various regions of the egg, and they observe an acceleration of development without any induction of a secondary nervous system. However, in one case Penners obtained a formation of a secondary embryo upon heating the ventral half of the blastula, but this is an isolated case and its significance is dubious.

Considering these results, we are forced to the conclusion that local heating is not sufficient to stimulate neural induction, for the few cases where a secondary medullary plate has been observed can be explained as a mechanical division of the organizer. The use of a thermal gradient, then, has not proved favorable to the idea that the organizer owes its inductive power to intense respiration.

There is, however, another means of attacking the same problem. This is to treat a ventral epidermal fragment which is a noninductor with a substance which increases metabolism. This experiment was first tried by Waddington, J. Needham, and J. Brachet\* who placed ventral explants in methylene blue, in cresyl blue or in 4,6-dinitro-*o*-cresol in low concentrations. After a few days the fragments were implanted into the blastocoel of a young gastrula, where at the end of gastrulation, they were found in contact with the ventral ectoderm, a position favorable for the induction of a secondary nervous system. The experiments were negative in the cases of cresyl blue and dinitrocresol but *the fragments treated with methylene blue induced a nervous system in seven out of thirty-eight cases* (Fig. 104).† Furthermore, the purely ectodermal explant self-differentiated to nervous system in three cases out of twenty-six. Thus it is clear that fragments of presumptive ventral epidermis which would never ordinarily have given rise to a nervous system can be transformed into neural plate when they have been treated with an agent which increases metabolism such as methylene blue. It was logical to assume that the dye stimulated chiefly the carbohydrate catabolism in the ventral explants and that this metabolism would then result in the formation of the inducing substance. In trying to imagine how this might

\* To be exact Brachet did not participate in this phase of the work which was chiefly Waddington's idea.

† Figures 104-107 are on Plates I-III.



occur, the most probable hypothesis seems to be the following: the ventral epidermis contains this inducing substance in the form of an inactive complex which breaks down when metabolism is stimulated. Now, this same phenomenon would be produced, in the physiological state, in the dorsal lip of the blastopore when the complex is destroyed during the process of invagination, when, as we know, glycogenolysis becomes intense. We will return later to this hypothesis. At present it is sufficient to say that Waddington, Needham and Brachet were careful not to exclude other possibilities. In particular it is possible to imagine a protein denaturation followed by a breakdown of the complex with a liberation of the inducing substance under the influence of methylene blue or a combination of this basic dye with nucleic acids.

This precaution has proved opportune. The question was taken up again in Needham's laboratory by Beatty, De Jong and Zielinski, who placed pieces of presumptive ventral ectoderm in a series of dyes and examined them later microscopically for the presence of a nervous system: methylene blue exhibited activity in three cases out of eight, but pyocyanine, which should greatly increase oxidation, produced no effect. Furthermore, Janus green and neutral red, which have no effect on oxidation, stimulated neuralization of the explants in a few cases. Beatty, De Jong and Zielinski verified the increase in oxidation with methylene blue at the active concentrations; it was moderate, not exceeding 45 per cent.

A parallel study was made by Boell and J. Needham. They followed the effects of dinitrocresol on anaerobic glycolysis in the organizer and in the ventral ectoderm of *Rana temporaria*, and reported an increase of only 16 per cent for the dorsal lip against 157 per cent for the ventral ectoderm. Now, it will be recalled, that dinitrocresol does not stimulate neuralization in ventral explants, so one must conclude that simple stimulation of carbohydrate breakdown is not sufficient to liberate the inducing substance and to produce neuralization. It will also be remembered that the recent research of Jaeger has clearly established that there is no cause and effect relation between glycogenolysis and induction.

It may be further noted that Finkelstein and Schapiro did not obtain induction by implanting into the gastrula agar impregnated with dinitrophenol. Thyroxine, which also increases respiration, likewise gave negative results. However, it must be added that

methylene blue, whose effectiveness in the neuralization of explants has been recognized by Ranzi and Tamini, Holtfreter, and by Pasteels (personal communication), does not induce nervous systems when dissolved in agar and then implanted. It appears, therefore, that the dye must be in solution to be active.

In summary, it is clear that methylene blue "neuralizes" ventral explants in 10 to 35 per cent of the cases, and at the same time increases the oxygen consumption by 45 per cent. However, the same effect is obtained by dyes that do not influence metabolism. Dinitrocresol stimulates anaerobic glycolysis especially in the case of ventral fragments, but it is nevertheless incapable of stimulating the formation of a nervous system. It must be admitted then that at the present time there is no weighty argument in favor of a direct connection between carbohydrate metabolism and induction. Actually, there is nothing to show that a simple increase in metabolism is in itself sufficient to cause the inducing power to appear. Child's ideas are only weakly supported on this point for methylene blue may act through a mechanism quite different from a rise in oxidation. The recent work of Holtfreter suggests that methylene blue is not specific in its action, but may be exerting an influence by reason of the necrosis caused by its toxicity, cells in a state of cytolysis being able to liberate some substances capable of stimulating neuralization in the explants of ectoderm.

Let us see now whether this lack of causal relationship between induction and metabolic changes is confirmed by the results of the action of *inhibitors* of metabolism on fragments of various origins. First of all, Waddington (cited by Needham) has observed that primary morphogenesis takes place when one subjects chick embryos, cultivated *in vitro*, to concentrations of cyanide which may inhibit metabolism by as much as 70 per cent. Brachet found (1939) the same result in *Discoglossus*. In this case, if the organizer is isolated with a part of the presumptive nervous system in the young gastrula stage and this explant is raised in a saline solution containing  $M/1000$  KCN, a beautiful nervous system will develop. Figure 105 shows this. As the cyanide was frequently renewed, it must be considered that the respiration was lowered by 80 to 90 per cent. Consequently, maintenance of normal oxidation is not a *sine qua non* condition for neural induction, the organizer retaining its full inductive power when it has only glycolysis and its oxidative reserve

as sources of energy. It may be mentioned further that dorsal fragments differentiate normally in the presence of malonate (Brachet and Rapkine), a poison that paralyzes succinic dehydrogenase and thus lowers oxidation in muscle. However, we do not yet know whether it depresses respiration in amphibian eggs.

We have also studied the effects of *inhibitors of carbohydrate metabolism* on the development of dorsal explants (organizer plus neural ectoderm) and ventral explants (ventral marginal zone plus ectoderm). Fluoride has no appreciable effect but iodoacetate gave some interesting results, the organizer showing a clear differential susceptibility in regard to this agent in concentrations of  $M/3000$  to  $M/10,000$ , in *Discoglossus* as well as in *Triton*. Although the mortality of dorsal explants at thirty hours was 78 per cent, in ventral fragments it was only 32 per cent. Thus we see that dorsal regions when explanted are much more susceptible to monoiodoacetate than the ventral parts of the gastrula.

It has also been attempted to determine whether the dorsal lip of the blastopore of gastrulae in which development was inhibited by monoiodoacetate remained inductive. A high percentage of perfect inductions (80 per cent) was obtained which cannot be distinguished from inductions given by normal organizers. But this fact has little real significance, since the action of monoiodoacetate is reversible to a large degree, making it doubtful if the inhibition of glycolysis is maintained after the tissue is implanted into the host.

The special susceptibility which the organizer shows to monoiodoacetate leads to the assumption that glycolysis plays an important role in morphogenesis, but the effect of this inhibitor on the carbohydrate metabolism of explants has not been studied and one cannot draw a definite conclusion as long as this gap has not been bridged. Another explanation of the sensitivity of the organizer to monoiodoacetate has been proposed by Rapkine (1938). This inhibitor may block  $-SH$  groups of proteins which in this case may take part in morphogenesis. The conditions under which Brachet worked (long exposure and low concentrations) make this hypothesis plausible, and it is also supported by the fact that fluoride, which does not react with  $-SH$  radicals, but does inhibit glycolysis, is not especially toxic for the dorsal fragments.

Thus Brachet and Rapkine have considered *the role of  $-SH$  groups in induction*. One can make these groups *disappear* by oxi-

dizing them with oxidized glutathione or alloxane or by combining them with iodoacetamide. If appropriate doses are chosen *an inhibition of the formation of the nervous system in dorsal explants* is obtained (Fig. 106). While the control explants show a closed nervous system, the treated explants show only a rudimentary neural plate, palisade-like in appearance. The oxidizing agents are, on the other hand, without action on the ventral fragments.

What happens when the concentration of —SH groups of the ventral explants is *increased*, which can easily be accomplished by treating them with cysteine, with glutathione in reduced state or with thiomalic acid, all of which contain an —SH group? Brachet and Rapkine obtained in 37 per cent of cases (twenty-six out of seventy) *the formation of a nervous system in ventral fragments* (Fig. 106). The sulfhydryl compounds thus behave like methylene blue in stimulating the production of a nervous system in regions that normally never form one. This result naturally suggests the idea that the —SH groups play a role in neural induction, perhaps in activating carbohydrate catabolism. However, Lindahl (1941) correctly argued that these sulfhydryl compounds merely produce a toxic effect with the result that the inducing substance is liberated from the breakdown of a complex. It will be recalled that cysteine is toxic in the case of the sea urchin egg. We will have occasion to return later to the role of the —SH groups in induction, but for the moment we shall simply state that the blocking of —SH groups inhibits neural induction while their addition to presumptive epidermis stimulates the formation of a nervous system.

The experiments just described have been criticized by Holtfreter, who was not able to reproduce the inhibition of the formation of the nervous system with glutathione or alloxane, but Holtfreter did not neutralize the acid solutions that he used and thus necessarily used concentrations of these substances that were much too weak to obtain the effect expected. On the other hand, the objection made by Lindahl and restated by Holtfreter that the inductions obtained by Bracher and Rapkine by means of cysteine and glutathione may be primarily due to cytotoxicity, appears legitimate.

Results analogous to those which Brachet and Rapkine obtained with cysteine have been noted by Ranzi and Tamini. These authors treated ventral explants with *Na sulfocyanide*, and observed 34.7 per cent induction of a nervous system, often accompanied by notochord

(22.3 per cent) (Fig. 107). This result agrees with the effects of sulfocyanide on the entire embryo. It will be recalled that this substance gives rise to embryos with a hypertrophy of the notochord and nervous system, and is thus a "hyperinductive" agent. The Italian authors were first of the opinion that, by analogy with the work of Lindahl, sulfocyanide stimulates carbohydrate metabolism and inhibits that of the proteins and that these new conditions increase the amount of inductor substance. This, of course, is merely a hypothesis which needs experimental verification. A study of the effects of sulfocyanide on the metabolism of amphibian eggs is in order without delay, just as is an analysis of the chemical effects of lithium. It is clear, moreover, that Lindahl has a valid criticism of the experiments of Ranzi and Tamini, namely, that there is no proof that sulfocyanide does not simply break down an inactive complex containing the inducing substance or does not cause a localized cytolysis. Ranzi and his collaborators now believe that lithium and sulfocyanide act on the viscosity of fibrous molecules, but the significance of their results remains obscure (see p. 324).

On the subject of lithium T. S. Hall has done some interesting work on the question of whether this agent acts directly on the organizer or whether it inhibits the ectoderm being subjected to the action of the organizer (reacting system). His experiments show that ectoderm treated with lithium reacts perfectly well to a normal organizer, while an organizer which has been subjected to lithium has its inducing power reduced. The action of lithium is thus essentially on the chordamesoderm, that is to say, upon the organizer. In addition, A. F. Burt has stated that lithium does not change the distribution of glycogen, but that it does retard the utilization of yolk.

It would be premature to attempt to interpret at this time all these facts because of the insufficiency of quantitative data. In general, the results show that various agents (methylene blue, thiols, sulfocyanide, etc.) stimulate neuralization of isolated ectoderm, and bring about local cytolysis, as suggested by Holtfreter. This latter fact contributes to the explanation of Barth's experiments where, under certain conditions, induction was obtained without the organizer (see p. 351). An increase in carbohydrate metabolism is not sufficient in itself to stimulate induction in the ectoderm and a lowering of the rate of oxidation does not prevent normal induction. The

question of whether induction is possible when carbohydrate breakdown is inhibited remains open.

### 5. Nature of the Inducing Substance

✓ The idea that the organizer owes its inducing power to a chemical substance has been accepted generally since the time when the dead dorsal lip was shown to retain its activity. It was in 1932 that Bautzmann, Holtfreter, Mangold and Spemann published their preliminary results. The method of killing the tissue was varied. Bautzmann used heat, while Holtfreter had recourse to drying, heat and freezing; Spemann fixed the dorsal lip in alcohol; and Mangold demonstrated that agar impregnated with an extract of the organizer was able to induce a nervous system.

Following this, progress was rapid because of the simplicity of the technique, which consisted in implanting a fragment of the dead organizer into the blastocoel of a young gastrula. This cavity becomes reduced during gastrulation (see Fig. 84) to a thin slit, and as a result the implant at the end of gastrulation is directly applied to the ventral epidermis, which "reacts" and is later transformed into a more or less well-formed nervous system. A second more difficult but more precise method consists in placing the dead organizer "sandwich-wise" between two fragments of ventral ectoderm.

✓ At the very beginning, Bautzmann and Holtfreter made a very interesting observation, namely, that the dead dorsal lip is not the only part of the egg which is capable of *induction*. If a piece of ectoderm, which in the living state is not an inductor, is killed by boiling and then implanted, it will induce a nervous system. Thus it suffices to *kill* the ectoderm in order to confer on it the ability to induce, a property which it naturally does not possess. Spemann, Fischer and Wehmeier have tried to explain this curious behavior by assuming that the ventral fragment contained an inhibiting substance which was eliminated upon killing the tissue.

This hypothesis had to be discarded, however, when Holtfreter, in a paper which aroused considerable interest, announced a series of unexpected results. He first showed that the dead organizer stimulates induction when wrapped up in a sheet of isolated ventral epidermis and that dead ectoderm or endoderm is no less effective than the organizer in this regard. Thus the factor responsible for induction can only be a chemical substance diffusing from the dead tissue.

Holtfreter also undertook to determine the properties of this inducing substance. He found that it resists boiling for an hour, treatment with alcohol, ether, xylol, 20 per cent hydrochloric acid for twenty hours. However, heating at 100°C. for an hour and a half or at 185°C. for one hour completely inactivates the inducing substance.

Even more surprising results were forthcoming. Nervous systems, often of considerable size, were formed when the *most varied organs*, dead or alive, were implanted into the gastrula. The tissues of invertebrates, however, were less potent than those of vertebrates, while yeast, starch and agar were not inductors at all. From this one must conclude that the active substance is widely distributed in animal tissues, being very abundant in the vertebrates, less plentiful in invertebrates, and lacking in plants. The negative results in the latter case probably are due to the presence of cellulose membranes which impede the diffusion of the substance responsible for induction. Indeed Ragosina and Toivonen have obtained mildly positive reactions after implantation of growing plant tissues, and Brachet obtained excellent results by implanting *yeast extracts*. Intact yeast, as we have just seen, is not an inductor, probably because of the impermeability of its membranes.

Holtfreter's observations may be summarized in the following table:

*Very powerful inductors:*

- Coagulated chick embryo extract.
- Liver, kidney, adrenal, heart, brain of mouse.
- Thyroid, kidney, liver, brain, tooth of man.
- Bottom layer of centrifuged brei of calves' liver.
- Liver of lizard, frog, *Triton*.
- Brain and retina of salamander.
- Heart, ovarian egg, muscle and liver of fish.

*Powerful inductors:*

- Lens of eye of mouse.
- Centrifuged calves' liver brei (middle and upper layers).
- Thyroid, kidney, liver, testicle, adrenal, fat of bird.
- Kidney and testis of lizard.
- Heart and limb bud of *Triton*.
- Frog muscle.
- Dragonfly ganglion substance.
- Lymph from larva of *Sphinx*.
- Limnaea* liver.
- Daphnia* extract.

*Weak inductors:*

- Blood, fatty tissue from mice.
- Liver, heart of salamander.
- Retina of *Triton* larva.
- Fatty tissue from dragonflies.
- Imaginal discs from caterpillar of *Vanessa*.
- Foot muscle of *Planorbis* and *Limnaea*.
- Subcutaneous muscle of enchytrids.

*Inactive inductors:*

- Ectoderm, endoderm of living gastrula.
- Tadpole gills.
- Starch, agar, egg albumin, pork fat, animal charcoal.

At about the same time E. Wehmeier\* published some similar results. She confirmed the inducing power of the dead organizer after killing with alcohol, ether, acetone, acetic acid. Heating at 90° for fifteen minutes did not alter it significantly. All of these physical and chemical agents brought out the inducing capacities in normally inactive ventral fragments. She also had success with implantation of brain, tumors, cartilage of rat, and the aqueous extracts of muscle, liver, thymus and pancreas also were inductive. The retina of the eye and especially the white and yellow of the egg are nearly without action. From this work it appears that there is no relation between the inducing activity and the rate of glycolysis in tissues. The retina, for example, with a high glycolysis, has weak powers of induction. Woerdeman (1933) reported at about the same time that muscle and tumors with high glycolytic rates are good inductors, but the table by Holtfreter and the experiments by Wehmeier show that carbohydrate metabolism is not a *sine qua non* condition for inducing tissue.

It may be mentioned here that Hatt observed very good neural inductions after implantation of the primitive streak of the chick embryo, while the remainder of the blastoderm showed little activity. Like Holtfreter and Wehmeier, Hatt noted that the results were better when the tissue was killed before implantation.

The final nature of the organs induced, which are often large and complex, has been followed recently by Chuang (1941). He

\* In reviewing these investigations and those following, we have not tried to follow a strict chronological order which would be detrimental to a clear exposition. The arguments, often vigorous, as to the question of priority, which have been raised, are already forgotten.



concluded that heterogenetic inductors (dead tissues) never give the normal structure of organs in regard to histological appearance and typical anatomical relationships, the implants merely stimulating new potentialities in the regions which they contact without insuring perfect morphogenesis.

To what can we attribute this state of affairs? It was J. Needham, Waddington and D. Needham who first drew attention to the inferiority of heterogenetic inductors as compared with the living organizer. These authors pointed out that the normal nervous system exhibits regional characteristics, forming a cerebral vesicle anteriorly, followed by a neural tube, the dimensions of which gradually decrease posteriorly. The picture is quite different after implantation of a dead organizer or some other organ, these heterogenetic inductors stimulating complex and abnormal organs to form. For example, a cerebral vesicle, a notochord and eyes may be found jumbled to-

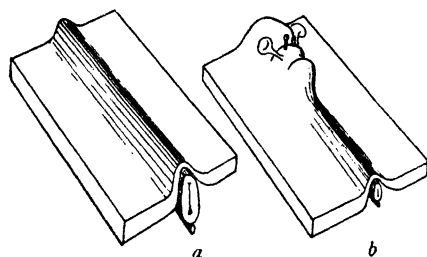


Fig. 108. Diagram showing the difference between evocation (a) and individuation (b) (Waddington and Needham).

gether. J. Needham, Waddington and D. Needham proposed to define the two reactions in distinctive terms, using the term *evocation* when the induction consists of an atypical mass and the word *individuation* when the host forms a typical organ differentiating normally along the embryonic axis. Heterogenetic inductors are thus evocators rather than organizers: the latter term should be exclusively reserved for the living dorsal lip of the blastopore, since it alone is able both to evoke the formation of a nervous system and to insure its regional organization, that is to say its individuation. The diagram (Fig. 108) will aid in understanding this necessary distinction.

We see then, that the problem is not as simple as appeared at first sight, and still a second factor enters to confuse the situation further. When an evocator is implanted, it undergoes chemical changes during sojourn in the host. If living tissue is implanted it

soon becomes autolyzed, and if a dead organ is introduced it may be rapidly attacked by the enzymes of the host. There is nothing to show, then, that the implanted evocators really contain the active substance, and indeed, the latter may arise secondarily by autolysis or digestion. Much evidence proves that this latter possibility may well be the case. Thus Finkelstein and Schapiro noted that autolysates of various organs, notably the hypophysis, are evocators after they have been incorporated into agar. In addition, Fischer observed that fragments of dead organs lost all their activity when they were covered with a layer of agar or gelatin which protected them from the action of the neighboring cells of the host. These facts lead one to suspect that the evocating agent, in such cases, is a soluble product of enzymic hydrolysis. Indeed, Brachet was able to demonstrate (1942, 1945) that the dead tissues were really attacked by the host, fragments of the organizer or of various organs fixed by boiling\* or by alcohol no longer giving the cytochemical reactions for thymo- and ribonucleic acids a few days after their implantation into the gastrula. There remains little doubt that the nucleases of the host have hydrolyzed the nucleic acids of the graft. It is very probable also that substances other than these nucleic acids undergo a breakdown. Indeed, the task of the biochemist who attempts to determine the exact nature of the inducing substance is singularly complicated.

These difficulties have not discouraged investigators, however, and work on the chemical nature of the inductor has appeared at an accelerated pace; the most important and numerous contributions have come from Needham, Waddington and their collaborators, but other essential points have been brought to light by Fischer and his students, Woerdeman, Barth, *et al.*

The first work of J. Needham, C. H. Waddington and D. M. Needham (1934) had considerable repercussions. The authors demonstrated, first of all, that extracts of ground-up neurulae were excellent evocators. When a brei of embryos was centrifuged, three layers were distinguishable, a fatty cap, a liquid containing proteins and fine granules, and finally a bottom layer of yolk. The implantation of the fatty layer and the yolk layer only rarely gave positive results, but the middle layer, after coagulation of the proteins by heat, gave good inductions in 16 per cent of the cases. The most

\* Recall that boiling for several minutes does not destroy all enzymes: ribonuclease in particular is not inactivated.

important result, however, was that an extract with ether or with petrol ether contained the active substance. If one evaporated the solvent and mixed the residue with agar or with triglycerides, both of which are inactive by themselves, induction was obtained in 8 per cent of the cases. The results were the same whether an ether extract of neurulae or of adult amphibian organs was used. The lipids remain active after saponification which led the authors to think that the evocator was a substance soluble in ether or petroleum ether, these properties suggesting a *sterol*. A few pure sterols were then tested for their evocating activity, but attempts made with cholesterol, oestrol, calciferol and phytosterol were negative.

These conclusions call for some comments. It must be noted, first of all, that the implants stimulate a very gradual series of reactions where all intermediates between simple local proliferation of the epidermis to a characteristic neural tube are obtained. It is not always easy to decide which cases are really positive and to distinguish them from the more ordinary "palisade" reactions. This point was recognized by the English authors who established a complete classification of the various types of reactions, but it appears that, at least at first, they considered cases positive which today would be doubtful. However, it is undeniable that authentic neural tubes were obtained after implantation of ether extracts, but the percentage of these was very low. Here is a portion of the statistics published by Needham, Waddington and Needham.

Nature of implant	Number of neural inductions	Number of experiments	Per cent of inductions
Adult tissues .....	5	10	50
Ether extract adult tissues .....	3	33	9
Aqueous extract embryos .....	10	63	16
Ether extract embryos .....	6	93	6.5
Unsaponifiable residue .....	3	23	13

The extract with ether does not therefore concentrate the active substance. Quite the contrary, these extracts are notably less active than the entire organ and are less effective than aqueous extracts of neurulae coagulated by heat.

The *quality* of the reactions does not increase following ether extracting. Some of the photographs published by Needham and

his collaborators are reproduced in Fig. 109\* and show the results obtained with aqueous extracts, petroleum ether extracts, and the unsaponifiable fraction of neurulae. Clearly the quality of the evocations diminishes in this series.

↪ It may be added finally, that these evocations are characterized by the complete absence of mesodermal inductions. Never do a notochord or somites appear although these structures are frequently found in experiments where normal organizers, or living and dead tissues are used as inductors. We will have occasion to return to this point.

In 1935 the role of the sterols in evocation was re-examined by Waddington, J. Needham, Nowinski and Lemberg. This time ether extracts of whole newts and of mammalian liver were used with success. The active substance was not saponifiable and could be precipitated by digitonin. Since it came out with cholesterol upon crystallization of the unsaponifiable fraction the evocator really appeared to be a sterol similar to cholesterol.

Here again the number of typical nervous systems evoked was small (seven cases in one hundred), but the authors explain this by a hypothesis of the quantitative factor operating in induction. According to this, depending on the amount of evocator substance present, a response will be obtained which will vary in size and structural complexity. The English workers confirmed by a long series of experiments that coagulated egg white was inactive. It will be remembered that Wehmeier had already recognized this fact, the experimental importance of which consists in the use of egg albumen as a convenient substance for implantation, since there can easily be mixed into it any substances which are to be tested for evocator activity.

Let us leave the above investigations for the moment and turn to some of those carried out in other laboratories. We have already seen that Woerdeman believed that he had established, by cytochemical methods, a tremendous glycogenolysis during gastrular invagination. It was this result that stimulated various investigators to see if the inducing agent might not be *glycogen*. The results were contradictory from the beginning. While Spemann, Fischer and Wehmeier obtained good inductions by implantating a mixture of gelatin and glycogen, Woerdeman and Holtfreter reported negative

\* Figures 109-113 and 115 are on Plates III-V.

results. Holtfreter, in addition, pointed out that chick embryo extract was one of the best inducing substances known but that there is no glycogen in the young chick embryo.

These facts suggest that the glycogen used by Spemann, Fischer and Wehmeier was contaminated with an active impurity. As a matter of fact, Waddington, J. Needham, Nowinski and Lemberg showed that an ether extract of impure glycogen gives good neural inductions in a number of cases. The quality of these inductions is good and the results are superior to those given by the unsaponifiable fraction of calves' liver or of entire Triton (Fig. 110). In addition, when the sterols from glycogen are precipitated by digitonin, a preparation that stimulates inductions is obtained. Its activity is very low, however (Waddington, J. Needham, Nowinski, Lemberg and Cohen).

These results, which were later admitted by Fischer and his collaborators, supported the idea of the lipid nature of the evocator. An important advance was made when Waddington and D. M. Needham showed that *synthetic polycyclic hydrocarbons* were also evocators. The secondary nervous systems were, however, generally atypical and small; an exceptionally good case is shown in Figure 111. However, the fact that synthetic substances, foreign to the living organism, are endowed with evocator activity is interesting, since in these cases it is not possible that impurities of a biological source are responsible for evocation. In addition, the "sterol theory" receives support since the hydrocarbons used are chemically similar to the sterols. Furthermore some of them have physiological activities analogous to those of sterol derivatives: two of the substances tested have an oestrogenic effect like sex hormones; two others are carcinogens.

The inducing activity of the ether extract of various organs has been confirmed by Barth. However, he believed that it was not a sterol that was involved but rather a phosphatide, *cephalin*. When this substance was isolated and implanted as a mixture with kaolin, inductions were obtained, but it was only effective in high doses (50 per cent) and the purity of the preparation used was doubtful. Waddington, J. Needham, Nowinski, Lemberg and Cohen showed that Barth's extracts remained active after saponification which destroys cephalin. In addition, lipids free of cephalin are evocators, so one cannot attribute the inducing power to cephalin but rather to an impurity, probably a sterol.

The work of Fischer, Wehmeier, H. Lehmann, Jühling and Hultzsich (1933–1935) occupy an important place because of the large number of substances which they tested for inductive activity. It is regrettable that they have published their results in such a summary fashion. The paper by H. Lehmann fortunately partially fills this gap. The German authors first established that the *inductive power of dead organs remains intact when they are freed from lipids* by long treatment with boiling fat solvents. Holtfreter drew the same conclusions from his own experiments (Fig. 112). However, Fischer and his collaborators confirmed the fact that the ether extract contained evocators but further found that their activity ceased when the extracts were neutralized with bicarbonate. These properties led to the belief that the substance responsible for inductions is an *acid* soluble in ether and in water.

The German workers had no success with implants of neutral fats, phosphatides, and the unsaponifiable fraction. On the contrary, *fatty acids* isolated from triglycerides were active, beautiful neural plates (Fig. 113) having been observed after implantation of *synthetic oleic acid*, which cannot be contaminated with sterols. Fatty acids were evocators only when they were liquids at room temperatures, since solid acids implanted as such never gave induction. They report only failures when they used the lower fatty acids and lactic acid.

But it is evident that fatty acids are not the only agents responsible for evocation by dead organs since the residue after removal of fats remains extremely active. What is responsible for inductions under these conditions? The experiments of Fischer and his collaborators show that *nucleoproteins* are involved, those from thymus (Fig. 113) and pancreas and liver being excellent inducers even after prolonged treatment to remove fats and purifications by several precipitations and by dialysis. The *nucleic acids* of thymus (Fig. 113) and of pancreas are also excellent inducers even after elimination of any lipids which they may contain. It was also determined that nucleic acids and nucleoproteins induce neural structures only when implanted in their natural state, their activity disappearing when they are embedded in agar. This leads one to suppose that nucleic acids must first be attacked by the enzymes of the host and transformed into simpler and more soluble derivatives, probably mononucleotides. Indeed, *adenylic acid from muscle* has been

shown to be a good inductor. Other mononucleotides tried were ineffective, probably due to their excessive solubility in water.

The authors concluded from these experiments that *only acid substances are able to induce the formation of a nervous system in the ectoderm*, and it makes no difference whether the acid is fat or water soluble. Induction by dead tissues, then, results from a *stimulation by an acid* of any sort (Säure-Reiz). Whether this mechanism plays a role in normal induction, however, is a question that cannot yet be answered.

Several additional facts may be gleaned from the work of H. Lehmann who found that the inductive power of ether extracts, shaken with water, disappeared completely and that some of the activity may be recovered from the aqueous phase. However, the potency of this fraction is decreased because the residue from evaporation is not easy to dissolve in agar. The unsaponifiable fraction could be concentrated nearly one hundred times, but, freed of all traces of acid, it remained completely inactive in more than one hundred experiments. Lehmann did not obtain inductions with certain carcinogenic agents such as benzopyrene and methylcholanthrene, but synthetic oleic acid at a concentration of 5 per cent stimulated induction in twenty-seven cases out of one hundred nineteen. Uric, hippuric, lactic, and phenylacetic acids were always impotent in stimulating the neural reaction, while indoxylacetic acid (auxin) gave one isolated positive reaction. Sodium and potassium soaps of oleic acid lacked all inductive powers. More recently Moricard and Gothié have obtained two inductions out of twelve cases after injection of sodium oleate into the blastocoel. Histological examination of these embryos showed that in the region of the oleate droplet large areas of degenerate cells appeared, and the authors themselves note that the secondary neural tubes generally arose near the more or less degenerate vitelline platelets. One must conclude that the effect of sodium oleate is probably secondary, this surface active substance producing local necrosis with the liberation of the inducing substances.

Moricard and Gothié (1945) have also shown that microinjection of bile salts into the blastocoel likewise gave positive reactions, while saponin, sex hormones, strong acids and bases, and detergents were inactive. They drew attention, at the same time as Holtfreter, to the presence of surface active substances in the amphibian egg,

and concluded from this that induction may be due to physical properties of certain substances which modify the morphogenetic movements by their surface active effect. But one cannot accept this explanation as valid at present because of the profound cytolysis produced by the substances injected.

To return to H. Lehmann's study of water soluble substances, nucleoproteins which were slightly acid and completely lipid-free were inductive in eleven cases out of fifteen. When thymonucleic acid was separated from histone by means of alkali, the nucleic acid retained much of the activity, inducing in twenty cases, out of thirty-nine (51 per cent) and the histone which is not entirely freed of nucleic acid was inductive in seven out of thirty-six cases (only 19 per cent). A thorough purification of the thymonucleic acid reduced its activity, but an alkaline hydrolysis, which increases the amount of free nucleotides, restored the entire activity. Although thymonucleic acid exerted no action when embedded in agar, adenylic acid from muscle remained active as an evocator under the same conditions. Good results were also obtained by the implantation of adenosine triphosphate adsorbed on a histone, but the percentage of inductions varied greatly from one series of experiments to the next. When the nucleotides prepared from any organ were subjected to increasing degrees of purification, a progressive decrease in activity is found, a phenomenon that may be due to the gradual elimination of the proteins, resulting in an excessive solubility of the nucleotides. Finally, guanylic and inosinic acids, inosine, zymonucleic acid, and a hydrolyzate of the latter have been tested with negative results.

Lehmann concluded that the inductive ability of dead organs does not reside in the lipid constituents but rather in their *nucleoproteins*, which give rise in the host to acid derivatives (mononucleotides?) responsible for induction by means of the *Säure-Reiz*. However, not every acid is an inductor, since certain physical properties, notably solubility, play an important role in this regard. Concerning the living organizer, Lehmann suggests that its acid production be investigated.

The results of Fischer and his collaborators are in complete contradiction on more than one point with those of Needham, Waddington and their school. The former claim that induction is a reaction showing little specificity, being stimulated by various acids, while the latter group maintains that the evocator is definitely a sterol. The



disagreement is particularly marked in the case of sterols as inductors, the German authors considering them inactive and refusing to recognize as inductions the "neuroid" reactions of the Cambridge investigators, attributing these to traces of acids in any case. This last suggestion has been rejected by Waddington, J. Needham, Nowinski, Lemberg, and Cohen, who state that the activity of the unsaponifiable fraction is not decreased when the acids that may be present are removed.

In order to clarify the situation, Waddington, J. Needham, and Brachet published a paper of chiefly theoretical significance in which they recognized a number of evocators which had been reported, including the unsaponifiable fraction, polycyclic hydrocarbons, thymonucleic acid, muscle adenylic acid, oleic acid, cephalin, and methylene blue. One may exclude cephalin which was apparently impure and then there remain the sterols, acids, and methylene blue.

If normal induction is thought of as mediated by a specific substance such as a sterol or an acid it can easily be imagined that various agents which release evocator activity in the ventral ectoderm (heat, acids, alcohol, drying methylene blue, etc.) cause a sterol or an acid to appear in the originally inactive material. Hence, numerous substances will be evocators because they *liberate the evocator from an inactive complex by an "unmasking" action.*

Do the acids unmask the sterols or is the reverse true? This dilemma is difficult to resolve, the question of the concentrations at which the substances act being all-important. The sterols and the polycyclic hydrocarbons are effective in concentrations of 0.1 to 0.2 per cent while the acids are active only at a concentration of 5 per cent, making it probable that their effect is an indirect one, injuring the surrounding cells and liberating the inductor from its complex.

In regard to the action of methylene blue, it may unmask the organizer by modifying the metabolism of the ectoderm, or by denaturing the proteins or again by acting directly on a sterol. Figure 114 schematically represents this concept.

What is the nature of the complex that contains the sterol responsible for evocation? The fact that desmoglycogen bound to proteins is rapidly utilized during gastrulation (J. Brachet and J. Needham) suggests the possibility that the evocator is combined in this same complex. There would thus exist a protein-glycogen-sterol complex which breaks down during invagination.

Nevertheless, this hypothesis, however tempting, is shown to be untenable, for we have already seen that the utilization of desmoglycogen is no more rapid in the organizer than in other regions (Heatley and Lindahl). Furthermore, Heatley, Waddington and J. Needham have demonstrated that desmoglycogen is perfectly able to produce evocation, and hence cannot be considered as part of an inactive complex. However, it is possible that desmoglycogen is attacked by the host and the complex in this way broken up. A cytochemical study of implants at the end of the experiment would resolve this problem, a point that appears to have been overlooked by the English workers.

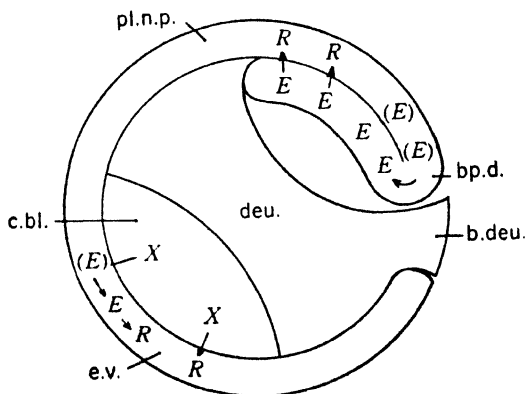


Fig. 114. Schematic representation of the hypothesis of Waddington, J. Needham, and Brachet of the liberation of the inducing substance from a complex: X, chemical substance implanted; (E), masked and inactive evocator; E, free and active evocator; R, response resulting in neuralization; c.bl., blastocoel; bp.d., dorsal lip of the blastopore; pl.n.p., presumptive neural plate; deu., yolk in endoderm; b.deu., yolk plug; e.v., ventral ectoderm. It is seen that the substance X may act directly or indirectly (Shen).

It will be remembered that although desmoglycogen like impure lyoglycogen is a good evocator it is not a complete inductor for it cannot stimulate individuation in the neural tube.

The problem of the role of the polycyclic hydrocarbons was reinvestigated by Waddington and by Shen: Waddington tried a large number of substances of various physiological activities taking care to implant them in very low concentrations (2 mg./cc.). Most of them exhibited activity and the quality of the inductions this time was excellent (Fig. 115). There was no direct relationship be-

tween the carcinogenic activity of the compounds used and their inducing power, certain substances devoid of biological activity evoking beautiful neural plates. Waddington's conclusions show much caution and some disappointment, since he thinks that it is very difficult to decide whether a substance acts directly, in the manner of the normal organizer, or whether it unmasks the inductor by liberating it from a complex. The idea that the concentration of the substance is of essential importance, the only theory left, is hardly unequivocal. Needham himself (1938) pointed out that in plants, synthetic heteroauxin is twice as effective as the normal physiological substance (auxin). The solution of the problem seemingly faces an unsurmountable obstacle, namely, the fact that one is trying to determine the activity of a substance on a material which already contains an evocator although it is inactive. The result is that we

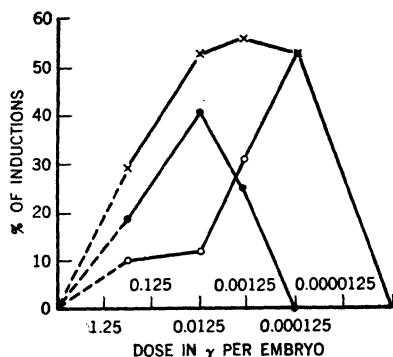


Fig. 116. Frequency of induction as a function of dose; x, induction of all types; ●, neural tubes; ○, palisade reaction (Shen).

can never determine the real nature of the inducing substance by implanting various chemical substances. Waddington ends by advising a renewed study of the living gastrula and a close comparison between dorsal and ventral regions.

Shen undertook an interesting *quantitative* study on the effects of a polycyclic hydrocarbon, 1,2,5,6-dibenzanthracene- $\alpha,\beta$ -endosuccinate, which has the advantage of being water soluble. It is a carcinogenic agent and a powerful evocator. Shen implanted this substance, incorporated into egg albumen in doses ranging from 0.0000125 to 1.25  $\gamma$  per gastrula. The results are shown in Figure 116 from which it is seen that there is a clear optimum at 0.0125  $\gamma$  per egg. This concentration compares with the order of magnitude which one would expect from a hormone or vitamin. The fact that

this substance is less effective at high concentrations implies that it does not exercise an indirect effect by liberation of the masked inductor. However, Shen reports that the embryos show pycnosis followed by cellular degeneration at the various concentrations which he used.

Later Shen showed that in eleven cases out of eighteen, neuralization of explanted presumptive epidermis was obtained when the tissue was cultivated in dilute solutions (0.001 per cent) of 1,2,5,6-dibenzanthracene- $\alpha,\beta$ -endosuccinate. However, here again there is every reason to believe that this substance exerts a toxic action, since even saline solution alone may produce necrosis in more susceptible species. Cytolysis forms one of the greatest dangers in experiments of this kind as Holtfrete has shown.

Let us terminate this discussion of the role of sterols in induction by adding that one of Waddington's students, Abercrombie, obtained evocation in the chick by use of the carcinogenic agent dibenzanthracene. Thus birds appear to react to sterols in the same way as amphibians.

In regard to the chemical mechanism of induction in the chick, an interesting study by Davis may be cited. This author subjected embryos between primitive streak and eight somite stages to monochromatic ultraviolet radiation of different wave lengths, and the embryos were then incubated for thirty hours and studied histologically. The radiation caused an inhibition of the closure of the nervous system without preventing growth. The photochemical efficiency curves are similar to the absorption spectra of sterols and the author concludes that substances of this type take part in the closure of the neural plate.

Before considering recent work of other laboratories, let us say a few words about the observations of Woerdeman (1933, 1936) which are still quite valid. This investigator reported that petroleum ether extracts as well as press juice of organs showing high glycolysis were good inductors, for example, muscle and tumors. A powerful carcinogenic agent, 1,2,5,6-dibenzanthracene, gave negative results. This substance had already been classified as having relatively low activity by Waddington and his collaborators. But the interest in Woerdeman's work lies chiefly in his pertinent discussion of the role played by the *host* in induction. He regards chemical agents as merely stimulators or activators, unable to create new po-

tentialities in the host, only being able to influence the various regions of the host by favoring a reaction. Now, the embryo can be subdivided into a series of fields. For example, the facility with which the ectoderm reacts to the optic vesicle to form a lens is not the same at all points, but decreases gradually with the distance from the lens primordium. It is the same with other regions, the presumptive neural plate reacting more readily with the organizer to form a nervous system than does the presumptive epidermis. These are fields which the evocator may modify to a certain degree, and Woerdeman considers that neural induction is only possible if the graft is near the nervous system of the host. This opinion, though extreme, is not without some justification. It is true that a heterogenetic inductor gives a better neural reaction if during gastrulation it comes to lie by chance near the nervous system of the host. The induced neural tube will be better developed in its anterior part as compared with more posterior regions just as with the normal nervous system. Nevertheless, one often obtains well-formed neural tubes at regions far from the host nervous system. We will return to this question in considering regional induction, emphasizing here the idea that the evocation reaction depends in part on the region of the implant. The more anterior and dorsal the implant is, the larger and better formed will be the induced nervous system.

With this idea in mind it is pertinent to discuss briefly the contributions of some Russian investigators. Finkelstein and Schapiro (1936-1938) did not obtain inductions after implantations of glutathione, cysteine and tryptophan mixed with agar, and negative results were also obtained with dinitrophenol, thyroxin and thyroïdin. It must be concluded then that it is not sufficient to raise the level of oxidation to induce the formation of a nervous system in the ectoderm. In four cases, Finkelstein and Schapiro observed a neural reaction after implantation of *carnosine*, a dipeptide of  $\beta$ -alanine and histidine found in muscle where its physiological role is poorly understood.

In addition, Balinsky, Goldstein, Lirzman and Schapiro made a large number of implants of ether or petroleum ether extracts of liver and of chick embryos, but only rarely did induction occur. Agar containing methylene blue gave no positive results which confirmed again the idea that local increase in metabolism is ineffective.

The experiments of Suomalainen and Toivonen, who implanted

a series of *vitamins* (A, B<sub>1</sub>, B<sub>2</sub>, C, D<sub>2</sub>) and *hormones* (adrenalin, thyroxin, heteroauxin) may be noted as well. A secondary nervous system never formed as a result of implanting these substances. *Yeast adenylic acid*, which was shown to be inactive in Fischer's experiments, gave rise to an isolated lens in a single case. This nucleotide was implanted in low concentrations (50 mg. per cent). The Finnish authors think that nucleotides are transformed by the host into co-enzymes which then stimulate carbohydrate metabolism.

There are also some results of Okada which might seem confusing unless they are interpreted properly. Okada implanted inert substances, such as Fuller's earth, silica and calcium carbonate, and obtained a number of weak inductions, finding no differences between acid, alkaline, or neutral implants. However, he obtained secondary nervous systems only when the implant changed the structure of the archenteron or stimulated a local necrosis. For example, the inert substances used by Okada might separate the roof of the archenteron mechanically and thus simply double the normal organizer. The Japanese author does not claim that evocation is not caused by an active substance but warns against new sources of error: the mechanical results of implantation of inert material and the danger of cytolysis.

The seriousness of this latter factor was also recognized by Barth (1939) and Barth and Graff. Barth (1934, 1935) maintained that the inducing agent was cephalin, but Needham, Waddington and their collaborators claimed that this phosphatide would not act by itself. This conclusion was not agreed to by Barth, however, who maintained that cephalin and the products of its hydrolysis were more effective than the sterols which are present as contaminants. But Barth was soon struck by the toxicity of cephalin and its tendency to produce cytolysis and this led him to investigate the effects of other toxic agents which may possibly liberate the evocator by injuring the neighboring cells. Among these substances *digitonin* must be included, which Waddington, Needham and their collaborators used to precipitate their sterols and which in low concentration (0.05 per cent) shows activity. Thus one may ask whether in the experiments of the Cambridge school it was a sterol or digitonin which really acted as the evocator substance. However, since digitonin is itself a derivative of the sterols, the seriousness of this objection is lessened. Barth also noted that egg albumen impregnated

with buffer mixtures from pH 3 to 9.7, can give rise to small neural plates, the acid or alkaline reaction stimulating a local cytolysis. Barth came to the conclusion that there was a similarity between induction and parthenogenesis, in that both phenomena are stimulated by a variety of chemical agents.

Barth has drawn attention to the facts, already noted, that the sterols induce in a low percentage of the cases, that the nervous systems induced are small and poorly differentiated, and finally, that the reactions are exclusively neural, since no mesodermal organs appear. It is certain that the dead and lipid-free organizer is much more effective than the ether extracts used by the English school. A comparison of Figures 109 and 112 will give convincing evidence of this.

These considerations led Barth to compare, on the same material, the activity of the ether extract and that of the residue. In the case of brain, Barth definitely concluded that the lipid-free residue is most effective. According to him, the best evocator is the precipitate obtained when gastrulae are extracted with NaOH at low temperatures and brought to pH 5 with acetic acid. This preparation, chiefly of protein nature, gives normally appearing nervous systems in a high percentage of cases. Thus Barth came to think of the agent responsible for induction as a *protein*, which if it becomes denatured, loses its ability as an inductor and becomes a simple evocator.

More recently, Barth and Graff have compared the activity of proteins isolated from neurulae by different methods, extraction with concentrated NaCl and precipitation by dilution, extraction by dilute NaOH and precipitation with acetic acid, and isolation of a protein gel by shaking with chloroform. Only the extract with NaOH showed good activity.

The interesting communication by Barth and Graff at the Symposium on Proteins at Cold Spring Harbor in 1938 was followed by a lively discussion between Barth and Waddington, which was chiefly concerned with the desirability of maintaining the hypothesis of an inactive complex in the ventral ectoderm. Barth claims that this supposition lacks support, since the implantation of chemical substances or heterogenetic inductors may provoke a local cytolysis, which stimulates the ectoderm in the same manner as in parthenogenesis. Waddington considers, on the contrary, that the hypothesis

of an inactive complex in the noninductor territories is still valuable even if it is not a demonstrated fact. It seems to us that the two ideas are reconcilable, since it is very probable that cytolysis stimulates the rapid breakdown of lipoprotein complexes. As a matter of fact, Holtfreter has shown that most of the lipids are present in the amphibian egg in the form of "lipochondria," that is, granules consisting of a mixture of lipids surrounded by a protein shell. Denaturing agents, cytolysis, and coagulation break this protein layer and liberate the lipid droplets. It is interesting to note that Holtfreter has observed the breakdown of these lipochondria during normal development. It may then be that conforming with Rapkine's hypothesis a denaturation of the proteins may be the basis of neural induction, of fertilization, and of cell division. Note that these same denaturing agents also liberate ribonucleic acid from the microsomes of the frog's egg (Brachet and Chantrenne).

Whatever may be the case, Barth's work has the double value of placing emphasis on the action of *proteins* and of drawing attention to the serious source of error, *cytolysis*. The role of the latter was examined by Cohen who used a thermocautery to produce local necrosis in the gastrula. He found that neural tubes and striated muscle sometimes appeared. The cause of these inductions might be found in a liberation of the normal inducing substance by cytolysis. One can ask, as Cohen does, whether the various chemical substances which have been implanted, the sterols in particular, do not derive their activity from the same mechanism. However, we must add that Margen and Schechtman were not able to induce a secondary nervous system by subjecting the gastrula to mechanical lesion. Nevertheless, Holtfreter's observations on explants cultivated *in vitro* in abnormal media have clearly shown the importance of cytolysis in this type of experiment.

Returning to the *possible role of the proteins in induction*, it was the definite localization of the —SH groups in the fixed egg and the fact that the reason for differential susceptibility of the organizer to monoiodoacetate might be found in its abundance of —SH groups (Rapkine), that led Brachet to formulate a hypothesis of the participation of these groups during induction (1938). This approach has the advantage of explaining why noninductor regions of the gastrula become evocators when they are killed, for during denaturation of the proteins there is an increase in the number of —SH groups.



Cytolyzing substances also produce a partial denaturation with liberation of free —SH groups. Viewed from the standpoint of this mechanism one can comprehend the effectiveness of the sterols and acids. Finally, there is a satisfactory parallel between the amount of —SH groups in various organs and their inducing power. In the particular case of the amphibian ovary, young oöcytes are better inductors than older ones (Holtfreter), and it is also true that their content in —SH groups is much higher. In oöcytes of large size, the germinal vesicle rich in —SH groups is distinctly more effective than the cytoplasm (Waddington, J. Brachet, 1938). In addition, we find that when the ovary is broken up and centrifuged, the middle layer is much more active than the lipid cap or the vitelline layer; it is also the only layer that gives the nitroprusside reaction.

This hypothesis, so attractive at first sight, meets with certain obstacles, however. For example, egg albumen is not an evocator although it has a considerable amount of —SH groups. In addition, there are the negative results of Finkelstein and Schapiro after implantation of cysteine and glutathione. A careful study of the question, carried out by Brachet and L. Rapkine as yet unpublished, has demonstrated that blocking of the —SH groups does not suffice to destroy the evocator action of the various protein fractions. Thus these radicals are not the agent responsible for evocation by heterogenetic inductors. However, we may not reject the idea that they may play a role in normal induction. Indeed, the experiments of Brachet and Rapkine summarized on page 320 argue in favor of the idea. However, we have the impression that —SH groups are necessary chiefly for the *reaction* of the presumptive neural plate to the inductor, and this opinion receives support from the fact that embryos in which the —SH radicals are blocked by iodoacetamide or chloropicrin form a notochord and normal somites but have only a rudimentary nervous system, often incompletely closed (Fig. 117).<sup>\*</sup> Thus it seems that the mechanisms which insure the thickening of the neural plate, its folding into a trough and its closure require the presence of —SH groups. Moreover, this conclusion does not appear to us to require modification because of Holtfreter's investigations which led to different results when he used oxidizing agents, since the concentrations in his experiments were too weak to give the desired effect.

<sup>\*</sup> Figures 117 and 118 are on Plates VI and VII.

We have seen above that the sulphydryl proteins of amphibian eggs are directly related, both from a morphological and biochemical point of view, to a *ribonucleic acid*. May it not be, then, the latter to which we must attribute the evocator ability of heterogenous inducers? All the arguments advanced for the role of the —SH groups are valid for this new hypothesis. For example, the failures after implantation of egg albumen, cysteine and glutathione become understandable, since these substances do not contain a nucleic acid. The later work of Fischer and his collaborators also lends support to our suggestion, since the evocative power of the nucleoproteins, the nucleic acid and the nucleotides has been amply demonstrated by their experiments.

Because of this promising outlook, Brachet thought it a good idea to submit this hypothesis to an experimental test (1942, 1944). At first he determined whether the active fraction obtained by Barth from amphibian eggs was really rich in nucleoproteins. The method of preparation made it probable that it was and determination of pentoses furnished the actual proof. Brachet then compared the ribonucleoproteins of the evocator from the point of view of their thermolability. Boiling for from one to two hours, which destroys the inducing activity of dead organs, extracts the ribonucleic acid in a quantitative manner, and the pentose content of the tissues drops to a very low value after heating to 150° C. for half an hour. Here again the resistance of the ribonucleoproteins and the evocator to heating is shown to be the same.

It will be recalled that the host is able to destroy the nuclear and cytoplasmic basophilia of fragments which have been killed and implanted; this is probably due to the host's action on the nucleic acids, which is rapid in the case of ribonucleic acid and slower for thymonucleic acid. This explanation is supported by the fact that amphibian gastrulae contain a ribonuclease as we have ascertained. When implants are placed in young gastrulae and the embryos later stained with Feulgen, there is frequently a relationship between the extent of the induction and the amount of thymonucleic acid in the implants; this phenomenon occurs most often in fragments of liver or young oöcytes fixed by heat or alcohol. In *Discoglossus*, out of sixty-two embryos examined, there was no case of induction without a great decrease in the thymonucleic acid content of the graft. Very often, the part of the implant most anteriorly located, and where in-

duction is always better, had less thymonucleic acid than the posterior region. Sometimes one is fortunate enough to catch the thymonucleic acid of the graft in the act of leaving the nucleus and diffusing toward the overlying ectoderm. The parallel between the success of induction and the loss of thymonucleic acid is not as good in *Triton* and *Axolotl*, but it is none the less striking, since it was found in one hundred and twenty-six cases out of one hundred thirty-nine. Even in the thirteen divergent cases it was observed that the ribonucleic acid of the graft had disappeared. These observations tend to show that an operation results in evocation only when the nucleic acids present in the implant are attacked by the host.

Another series of experiments by Brachet furnished results which are even more conclusive. Fragments of dead organizers, which were first *freed of their nucleic acid* by a two-hour treatment with *ribonuclease* at 65°, were implanted; the controls were subjected to water at the same temperature for the same time. After this digestion with ribonuclease inductions dropped from 67 per cent (forty-four cases out of seventy-four) to 20 per cent (seventeen out of seventy). A subsequent study showed that the enzyme used contained a large amount of soluble derivatives of ribonucleic acid which impregnated the graft; they could be responsible for the few inductions that persisted after enzymic hydrolysis. When crystallized ribonuclease was used in place of the impure enzyme, no neural induction appeared in twenty-four cases although nervous systems appeared in thirteen out of twenty-nine control embryos. Thus the percentage of successful induction dropped from forty-five to zero after the action of the enzyme.

The idea that dead organs owe their evocative activity to their ribonucleic acid begins to take form. If such is the case, we would expect that nucleoproteins of varied origin would be active and that the inductions would be most frequent when *the amount of ribonucleic acid in the graft was highest*. In order to isolate nucleoproteins Brachet resorted to a different technique from that of Fischer. To avoid the dangers of acidification, the nucleoproteins were prepared by ultracentrifugation according to the principles outlined in Chapter VI, the risk of a *Säure-Reiz* being thus reduced. In order to vary the ribonucleic acid content of the implants the proteins of the supernatant liquid after ultracentrifugation were sometimes implanted,

since these proteins have a very low pentose content. In other experiments, the layer of ribonucleoproteins was treated with crystallized ribonuclease to remove nucleic acid. Brachet was careful to use some ribonucleoproteins of plant origin (yeast), since it had been claimed that plants, yeasts in particular, were free of evocator substances. This assertion certainly could not be correct if a ribonucleoprotein was responsible for evocation. Finally, thanks to the kindness of Professor Manil, it was possible to use a ribonucleoprotein purified by repeated crystallization, the tobacco mosaic virus, which would hardly be contaminated by foreign substances.

The table below summarizes the results, while Figure 118 reproduces one of the evocations obtained.

Nature of implant	Number of evocations	Total experiments	Per cent evocation	Ribonucleic acid of implant in %
Liver granules .....	36	52	69	1
Tobacco mosaic virus .....	26	37	70	1
Yeast granules .....	10	16	62.5	1.1
Kidney granules .....	15	28	53.5	0.8
Gastrula granules .....	13	35	37	0.4
Supernatant fluid, kidney .....	9	38	24	0.1
Supernatant fluid, gastrula .....	6	31	19	0.2
Supernatant fluid, liver .....	5	47	10.5	0.1
Liver granules + ribonuclease .....	3	58	5	trace
Tobacco mosaic virus + ribonuclease	0	20	0	trace
Serum proteins .....	0	10	0	0

Granules = bottom layer after ultracentrifugation fixed in alcohol.

Fluid = fluid from ultracentrifugation precipitated with alcohol.

It is seen that the frequency of evocation is, in large part, proportional to the ribonucleic acid content of the implants. For example, the supernatant fluids after centrifugation are poor in ribonucleic acid and are regularly less active than the granules. It is also true that treatment with ribonuclease almost completely suppresses activity, the total of our experiments with the purified enzyme giving only three inductions out of one hundred and two (3 per cent) against sixty-four out of one hundred and four (61 per cent) for the controls. Various assumptions could account for the persistence of induction after the action of ribonuclease: an incomplete action of the enzyme, or presence of other evocator substances such as sterols, or cytolysis of the neighboring cells. During cytolysis of the gastrula, we see first an increase and soon after a disappearance in baso-

philia. Hence we may conclude that at this time the amount of ribonucleic acid in the cells increases and then later decreases.

It is noteworthy that the nucleoproteins of the gastrula are no more effective than extracts of other organs. In addition, it is a well known fact that the dead organizer is not as good an evocator as other tissues. It is also remarkable that yeast and tobacco mosaic virus are extremely powerful evocative agents, although these preparations are obviously not of animal origin. Furthermore, we have since (1945) obtained typical neural inductions with a nucleoprotein isolated from wheat embryos (triticonucleoprotein).

It is evident, however, that it is not necessarily ribonucleic acid itself that is the inductor. We know that this acid is hydrolyzed into more diffusible derivatives of lower molecular weights when acted upon by the ribonuclease of the host. It is plausible to assume that one of the nucleotides may be the active factor, as Fischer has already done. Unpublished observations made by Brachet and Rapkine indicate that certain nucleic acid derivatives are evocators in low concentrations, but further investigation will be necessary to solve this question. It would be premature to try to decide whether these nucleotides act by themselves as the evocator substance or whether they simply form an acid stimulus. Indeed, one cannot exclude the possibility that nucleic acid or the nucleotides liberated from the nucleoproteins of the graft merely have a cytolyzing action on the surrounding ectoderm. Proteins freed of their nucleic acid by ribonuclease would in that case be devoid of a cytolyzing action.

Because of the growing importance assumed by the problem of cytolysis resulting from the work of Barth and Holtfreter, it was of interest to know the behavior of nucleic acid during cytolysis. Brachet has undertaken such a study, still in a purely cytochemical stage, which consists of an examination of ectodermal fragments, unfertilized eggs, and blastulae in various stages of cytolysis, using the Feulgen reaction and staining with toluidine blue (the specificity of the latter is controlled by the use of ribonuclease). When one places ectodermal fragments in a physiological solution free of calcium or slightly alkaline as in Holtfreter's experiments, the injured cells are distinguished by a decided increase in basophilia which is sensitive to ribonuclease. Usually there is a segregation of the cytoplasm from the yolk, as in a centrifuged egg, but sometimes entire cells become strongly basophilic. When the cells enter a more advanced

stage of cytolysis and begin to break away from the fragment, the basophilia of the cytoplasm disappears completely while the nucleus remains clearly Feulgen positive. Thus it seems clear that during cytolysis ribonuclease (which is present in the explants) attacks the ribonucleic acid of the cells which then behave exactly like implants of a ribonucleoprotein nature which lose their basophilia during their contact with the host. These observations suggest the possibility that the mechanisms for neuralization in ectoderm explants in a state of partial cytolysis may be identical with those which operate during grafting of dead organs or nucleoproteins. In addition, during the cytolysis of unfertilized eggs left to themselves, one observes a separation of the ribonucleoproteins and yolk followed by a disappearance of basophilia.

The experiments we have just summarized prove, to our way of thinking, that the evocative ability of heterogenetic inductors, in particular that of *dead organs*, is due to their *nucleic acids* which undergo a degradation in the host. However, nothing as yet permits an extension of this conclusion to the living organizer, which we will discuss in the last chapter. We have now to consider the facts at our disposal regarding regional and specific inductions.

### 6. Specificity of Inductors

We have already emphasized several times the fact that the living organizer induces a complete secondary embryo, containing a nervous system with regional differentiation as well as mesodermal structures. The addition of the living organizer to a layer of ventral ectoderm gives the same results (Holtfreter).

Spemann very quickly came to dissociate the head organizer from the trunk organizer. This distinction was confirmed by Hall's experiments and the problem was re-examined by F. E. Lehmann. When the young gastrula is treated with lithium at the time when the head organizer invaginates the latter is especially affected and lesions of the brain and sense organs result. At the end of gastrulation, during invagination of the trunk organizer, lithium influences the morphogenesis of the trunk and tail chiefly. However, Lehmann's conclusions cannot be accepted without certain reservation, as has been pointed out by Pasteels, who has recently devoted an important study to the effects of lithium on development. It seems clear that the material which has already invaginated in the advanced gastrula

is protected to some extent from the effects of lithium. This is notably the case for the head organizer, which will no longer be exposed at the end of gastrulation. This protection, however, is shown only in *Triton* and not in the frog.

Questions concerning the chemical nature of the agents responsible for various inductions came up at this time: is the difference between the head and trunk organizer quantitative or qualitative in nature; must we consider that distinct substances participate in induction of the nervous system and the mesodermal organs, respectively; and, finally, must we assume that the morphogenesis of each sense organ (eye, ear, nose) is under the control of a chemical substance for its formation?

A partial answer to these questions may be found in the very interesting papers that Chuang, Toivonen, and Barth and Graff have recently published. We will here discuss only the essential points, beginning with the work of Barth and Graff, the only authors who have tried to test for possible chemical differences between head and tail organizers. The work of Chuang and of Toivonen, on the contrary, pertains to abnormal inductors (dead organs).

Barth and Graff compared the activity of extracts of the anterior and posterior roof of archenteron in the neurula stage on presumptive epidermis. In the first series of experiments they used an alkaline extract and precipitated the proteins with acetic acid. Next they placed fragments of ectoderm in contact with anterior and posterior roof of archenteron of neurulae which had previously been frozen and dried at low temperature. But in neither case did they obtain qualitative differences in the nature of the induced nervous system.

Chuang (1938) placed explants of the ventral ectoderm in contact with fragments of *Triton* liver and mouse kidney. Liver was a powerful evocator which nearly always induced *trunk* structures: notochord, muscle somites, and posterior nervous system. The kidney, on the contrary, induced chiefly *cephalic* structures, neither notochord nor muscle being found but rather brain and sense organs. The fact that the two organs used show a certain specificity indicates that the nature of the evocator is not an indifferent matter. Chuang, as a matter of fact, worked under conditions which reduced the host effects to a minimum, whether these effects are due to an axial gradient or a "field of individuation" (Weiss) which imposes a definite structure upon the various regions of the embryo.

Chuang continued his work by implanting the same organs into the *entire gastrula*. Under these conditions we must consider the influence of the host, and, in fact, the specificity of the heterogenetic inductors was diminished. In this case the kidney induced some mesodermal structures although the evocations were still mainly of cephalic structures. The region of the host where the implant finally is located at the end of gastrulation is of course of importance and this second factor always tends to obscure the specificity of inductors. The investigations of Chuang answer in part the doubts raised by Woerdeman as regards the real role of evocators, discriminating as they do between that part of the reaction which stems from the implant and that which is a function of the host.

Chuang also analyzed the effect of *heat* on mouse kidney. If this organ is subjected to brief boiling, its inducing power increases, while the tendency toward the evocation of mesodermal structures is increased; but heating at 100° C. for five minutes *completely abolishes the inducing capacity for mesoderm without reducing the frequency of neural inductions*. After fifteen minutes of boiling, the mesodermal inductors have entirely disappeared, evocation of brain becomes less frequent, and the sense organs appear in greatest number. Heating for one hour leaves only the power to induce sense organs.

Chuang concludes from these tests that mesodermal organs are induced by a *specific* substance, qualitatively different from the neural inductor and that inductors can be distinguished from each other by their unequal thermolability. Similarly, it is probable that the sense organs are formed by the action of a specific substance, distinct from the preceding two.

Chuang reported his last results in 1940. First, he made certain that heating of the kidney to 100° C. for two hours destroyed all inducing power. He then investigated the effect of heat on *Triton* liver, which is chiefly an inductor of trunk mesoderm in the living state. Boiling for a few seconds suffices to cause the evocation of mesodermal organs to disappear; neural inductions are also rarer while those of sense organs increase. After five minutes at 100° C. the induction of nervous systems is better and more frequent, without the reappearance of mesodermal organs. Finally, after fifteen minutes of boiling, embryos are obtained in a few cases with accessory brains, but one often finds sense organs as well. These results



confirm the author's opinion that the nervous system, the mesodermal organs and the sense organs form under the influence of specific substances for each group of structures.

Toivonen arrived at similar conclusions at the end of an extensive study on the inductive power of a number of organs, killed with alcohol (liver, kidney and thymus of several vertebrates). According to the situation, there was obtained either a telencephalon with nasal placode and eyes exclusively, or posterior head structures (rhombencephalon and mesencephalon with otocysts). The kidney of the guinea pig and perch stimulated mesoderm solely, although this organ, used fresh from the rat, is a neural inductor (Chuang). However, the mesoderm formed by implantation of these organs induced in turn a nervous system composed of a rhombencephalon or medulla. The action of thymus of the guinea pig deserves particular attention since it stimulated specifically the formation of an isolated lens in absence of the optic vesicle. Toivonen concluded that the host exercises a regional influence on the responses to an inductor, but that it is weak enough so that the specificity of the evocators is still apparent. Toivonen believed, as Chuang did, that different organs are induced by qualitatively different substances. The surprising specificity of the thymus, an organ rich in nuclei, may be correlated with the fact that a nucleotide, adenylic acid, induced an isolated lens in the experiments of Suomalainen and Toivonen.

This last point was re-examined by Rotmann who obtained very different results from those of Toivonen after implanting guinea pig thymus. In 24 per cent of his cases he found the formation of accessory tails, often with notochords, somites, and pronephros. This so-called lens inductor is thus able to function as a caudal inductor and stimulates the formation of mesodermal structures. In the material already studied histologically at the time of publication of the paper, Rotmann had not found a single case of an isolated lens. This investigator thought that the question of whether there are *specific* inductors or whether the host reacts specifically to *quantitative* differences of the same substance is still open, and correctly pointed out that inductive substances can only be liberated sometime after implantation following reactions between the host and the graft.

The work of Lopashov, Van Cleave, and Mikami on the formation of sense organs may be considered in light of these last conclu-

sions. Lopashov implanted dead optic vesicles into the blastocoel and found that they induced optic vesicles and lenses in the host. However, upon grafting nasal placodes and otocysts, he did not find these structures induced. Van Cleave studied chiefly the action of dead optic vesicle on ectodermal *explants*. In a few cases he obtained optic vesicle accompanied by lens, this result agreeing with those of Lopashov. Van Cleave then determined that the ether extract of optic vesicles is inactive. According to Mikami, implantation of dead optic vesicles only rarely leads to induction of this same organ. More recently, Van Cleave (1947) has studied the effect of optic vesicle, of posterior brain, and of liver of advanced larvae, which were first fixed in alcohol, on explants of ectoderm. Optic vesicle and posterior brain proved to be much more active evocators than the liver of older embryos, but in no case was a specific reaction obtained.

These authors believed, as Woerdeman (1939) did, in the existence of specific substances responsible for induction of sense organs, but this opinion, also shared by F. E. Lehmann, is not universally admitted. Reith, in particular, devoted a long critical study to the question and believes that the differences between cephalic and caudal inductors are probably of a quantitative nature and coupled with the cephalocaudal gradient. Such an interpretation is in agreement with the theoretical ideas of Dalcq and Pasteels who do not consider it necessary to appeal to specific inductors to explain morphogenesis. It is clear that some of the observations by Pasteels, notably the appearance of notochordal nodules in the midst of the nervous system of embryos centrifuged in the blastula stage, are hardly compatible with the idea that different substances are responsible for the formation of notochord and nervous system.

One might have hoped that the work of Shen, the only author who implanted a definite chemical substance in varying concentrations, would bring a clear answer to these points in dispute. However, he obtained only neural inductions without any mesodermal structures. Now, this observation is in favor of the idea of specific substances, but it may be asked, as Rotmann did, whether the embryos examined by Shen were cultured long enough for mesodermal organs to develop. It would certainly be very interesting to repeat Shen's experiments and to try to obtain a longer survival of the operated embryos.

As to the fragments of ectoderm cultivated by Shen in solutions

of carcinogenic hydrocarbons, they show, with regard to neuralization, merely balls of nervous tissue without organization into tubes, and sense organs and mesodermal derivatives are completely lacking in spite of prolonged culture. It appears clear in this case that the evocator had a purely neural action.

The problem of the specificity of inductors remains very obscure at present. The results of Chuang on the differential sensitivity of the neural and mesodermal inductors to heat and those of Shen—although the future may change them—lead one to think that we are here dealing with variations of a qualitative nature. The fact that a *brief* heating profoundly modifies the character of the evocator would suggest that the nature of the proteins enters into the situation. It is possible to envisage as a hypothesis that the inducing substance could be a conjugated protein, a nucleoprotein, for example. Nucleic acid would play the role of evocator, stimulating the ectoderm, while the protein would confer specificity on the reaction. This would account for the fact that the denaturation of the protein by heat would alter the character of the induction.\* Reith also observed a loss in regional specificity without the disappearance of inductor capacity by irradiating the isolated organizer with ultraviolet light at 2800 Å, a wave length that has a more marked effect on proteins than nucleic acids. The gradual loss in activity due to prolonged boiling is best explained by progressive extraction of nucleoproteins, a fact demonstrated by Brachet. These assumptions show certain analogies to an interesting concept developed by Waddington in 1938. The English embryologist thinks that normal induction may be the result of the collaboration of two factors: the primary evocator, plus “modulators” which modify its action and bring about regional differences. In the above hypothesis nucleic acid would be the evocator and the proteins to which it is joined would be the modulators. It is clear that the proteins, as Barth and Graff pointed out, are the chemical components which are best fitted for explaining development on a chemical basis by reason of their complexity and their extreme specificity.

It would be of little value to discuss these interesting ideas,

\* However, it must be noted that the change in specificity produced by brief boiling can have a different explanation. The implanted organ in the living condition undergoes autolysis lacking in the graft which is first boiled; the possibility of the origin of specific inducing substances during the course of autolysis cannot be neglected.

which are still inaccessible to biochemical analysis. More progress must be made along biochemical lines before such hypotheses can be adequately evaluated.

### 7. Discussion and Conclusions

The most important discoveries discussed in this chapter may now be examined critically.

Among recent findings two salient points emerge which attract attention. First, induction is stimulated by a *chemical substance* and second, the living organizer is the site of a *special metabolism*, chiefly carbohydrate. In fact it appears clearly demonstrated that in the region of the dorsal lip of the blastopore, carbohydrate catabolism first begins. Measurements of the respiratory quotient, carried out in various laboratories with different techniques, have given results which are in good agreement, so that the anaerobic glycolysis and intense glycogenolysis of the organizer are no longer in doubt. Concerning oxygen consumption, it seems clear that the organizer is not the region of highest respiratory activity in the egg, but rather that it is the presumptive nervous system of which this is true. However, oxidation in the dorsal lip of the blastopore is greater than in the corresponding ventral regions which have the same content of yolk.

The nature of the inducing substance is obviously a subject of paramount interest. Above all, the accumulation of obstacles which has distracted progress in this field must be emphasized. For example, chemical agents may substitute for the organizer in an indirect manner by liberating the inducing substance from a complex or by cytolyzing neighboring cells. Then, the presence of a graft may also disturb gastrulation and bring about a division of the roof of the archenteron, and furthermore the substance implanted may be modified by the host: we have seen how the nucleic acids undergo degradation and Suomalainen and Toivonen think that oleic acid, which is active according to Fischer, undergoes a transformation into a sterol. Finally, in judging the specificity of the implant, we must not underestimate the role of the host regional differences.

With these reservations, however, it would be absurd to think that "anything" is an inductor. Agar, for example, is inactive and egg albumen practically so, and among artificial inductors there are good ones and poor ones. Some regularly give excellent reactions;

others only act rarely and stimulate atypical structures such as "neuroid" tubes or "palisade" plates. It would certainly be a mistake to interpret the results of Okada's experiments on the implantation of inert substances as showing that these substances are in themselves active, since they certainly liberate the normal inductor secondarily by means of a cytolytic action. There is thus no need to adopt an unjustified pessimistic attitude.

We are confronted with two concepts at present, the nonspecific acid stimulus (*Säure-Reiz* of Fischer) and the sterol theory (Needham and Waddington). In what direction is the balance of evidence at this time?

The results of Fischer and his collaborators can be explained if we claim, as do Needham and Waddington, that acids are not active except in high doses and that they liberate the sterol evocator from a complex which in itself is inactive. It is important to note that, according to Fischer and H. Lehmann themselves, certain conditions of solubility and diffusibility, as yet poorly defined, must be fulfilled before an acid will act. The idea that all evocation is the result of an acid is hard to defend. Waddington, Needham, and their collaborators have many times taken care to neutralize the sterols and the hydrocarbons which they implanted, and their results were not altered by this procedure. Also, according to Barth, egg albumen impregnated with a buffer at pH 3.0 gives very small medullary plates and no neural tubes, while the injection of acids into the blastocoel gave no inductions in the experiments of Moricard and Gothié. Nucleoproteins isolated by ultracentrifugation, which eliminates the use of an acid, are perfectly active. Thus it appears doubtful that neutral or slightly acid implants owe their potency to an acid stimulus. As for acids implanted at high concentration, they probably produce cytotoxicity and may therefore liberate the normal inducing substance. Holtfreter also attributes the increased frequency of neuralization in explants cultivated in a slightly acid saline solution to cytotoxicity.

Coming to the sterol theory, the Cambridge school has demonstrated beyond doubt that certain polycyclic hydrocarbons are excellent evocators even in low concentrations. The proof that heterogeneous inductors owe their activity to a sterol has not, however, been established in a convincing manner. The unsaponifiable fraction isolated by the English investigators always seems to be much less

active than the residue, and the extraction and purification of the sterols from an organ does not lead to a concentration of the active agent. A serious obstacle also is the fact that dead tissues after prolonged extraction with fat solvents retain their complete inducing power. Finally, the question of concentrations has only relative value, as Waddington and Needham admit. Certain recent observations by Töndury lead us to think that even the most powerful polycyclic hydrocarbons act indirectly without substituting for the normal organizer. In fact, Töndury has stated that a number of derivatives of the sterols, the sex hormones especially, cause disturbances in mitosis and abnormalities in development at extremely low dosage (1:100,000 to 1:1,000,000). Briggs and Briggs have also declared that in weak doses a carcinogenic hydrocarbon, dibenzanthracene, produces a number of degenerative phenomena in the cells of embryos of *Rana pipiens* without any specific change in any region. If indeed these substances are toxic at such low concentrations, the possibility of cytolysis by the hydrocarbons thus far studied, even at great dilution, must be considered. We believe that under these conditions it is decidedly premature to consider the active substance of the organizer as a sterol. We wish to make it clear that we consider a sterol as a possible chemical agent in evocation but that the demonstration of this hypothesis appears incomplete.

The observations of Fischer, of H. Lehmann, and of Brachet make it very probable that *dead tissues* are evocators because they contain nucleoproteins which the host breaks down little by little. Nevertheless, this is not evidence in itself for the chemical nature of the normal inductor. In any case, the nucleoproteins are just as good inductive agents as the most powerful hydrocarbons and can be shown to be superior to ether extracts and especially the unsaponifiable fraction. The work of Barth and Graff and of Balinsky and his collaborators are fresh proof of this.

From the discussion thus far it appears that the nature of the active substance in the *living organizer* will be very difficult to ascertain. The method of implantation which is so widely used at present probably will not give the solution, since, as Waddington has so judiciously remarked, it will always suffer from the fact that one runs the risk of liberating the active substance by cytolysis or by a breakdown of a complex. The effect of concentrations is of little interest unless it is a matter of comparing the effectiveness of similar

substances with each other, substances differing little from the point of view of their toxicity. A return to the study of the living organizer is thus necessitated, from all the evidence. As Barth has remarked, in order to identify the inducing substance, we must demonstrate its presence in the roof of the archenteron and prove that it stimulates specific structures.

Our knowledge of the localization and the metabolism of evocator substances during gastrulation is still too fragmentary to draw definite conclusions. The metabolism of the sterols in the amphibian egg unfortunately remains completely unknown. We are a little better informed with regard to acid production, however. As we have seen it is large enough to be measurable manometrically only under anaerobiosis and is most intense on the dorsal side. The measurement of  $\text{CO}_2$  chemically combined during the determination of the respiratory quotient reveals no striking differences between dorsal and ventral explants if one can judge from the results of the experiments by Boell, Koch and J. Needham and those of Brachet. This fraction should decrease if an acid is formed in any quantity. However, it will be recalled that the pH of the vegetal pole, with much yolk, is less than that of the animal pole according to Dorfman and since the organizer is fairly rich in yolk, it may be that it is more acid than the presumptive nervous system with which it comes in contact. Precise measurements of pH in the various regions of the egg have been announced by Dorfman but have not as yet been published so far as we know.

We are better informed with regard the localization of nucleoproteins in the normal gastrula. We have seen above that cytochemical techniques and pentose determinations have shown that the organizer is richer in ribonucleoproteins than corresponding ventral regions. Invagination apparently is accompanied by a decrease in the amount of ribonucleic acid, but we cannot be positive because of the lack of quantitative measurements. It is clear that the interface between the roof of the archenteron and the presumptive nervous system is particularly rich in ribonucleoproteins at the end of gastrulation, so important exchanges between the inductor and the reacting material must certainly exist at this time. Perhaps the nucleic acids of the organizer become hydrolyzed into mononucleotides during invagination and pass into the neural ectoderm where they take part in the synthesis of nucleic acid. According to such an hypothesis,

a situation identical to that which occurs after implantation of dead tissues would be found. There is no doubt that the nervous system becomes richer in ribonucleic acid at the time of its formation and that this substance is distributed in gradient form following the "morphogenetic potential" of various regions.

Some authors (Waddington, 1939; Reith) have put forth the hypothesis that artificial and natural evocators stimulate a special metabolism in the ectoderm. It is clear that one of the manifestations of this metabolism is the synthesis of ribonucleoproteins which possibly results in the synthesis of proteins. This synthesis of ribonucleoproteins is particularly apparent in the case of double embryos obtained by centrifugation at the blastula stage (Pasteels and Brachet); the nervous system of the secondary structures is characterized by an intense basophilia which may even exceed that of the primary nervous system. Is the formation of ribonucleoproteins stimulated by a sterol derivative? This is possible, for Graffi has shown that a carcinogenic hydrocarbon, benzopyrene, selectively accumulates on granules rich in those ribonucleoproteins which are sedimented and isolated by the ultracentrifuge and which are found in all tissues. These granules are normally present in amphibian eggs as Brachet and Chantrenne have demonstrated, and it is very probable that in the gastrula, too, it is these particles, rich in nucleoproteins, which take up the carcinogenic hydrocarbons. In addition, it is interesting that these granules strongly adsorb another evocating substance, methylene blue. It could well be that changes in these particles, under various influences, affect the synthesis of nucleoproteins and secondarily that of the proteins.

Furthermore, the synthesis of ribonucleic acid in yeast is directly coupled with carbohydrate catabolism; inhibition of the latter rapidly depresses synthesis (Jeener and Brachet). Conversely, we know that a number of the derivatives of ribonucleic acid take part in the metabolism of sugars and certain indications even show that yeast nucleic acid is the precursor of important coenzymes of oxidation and of the fermentation of sugars (Ostern and collaborators).

The point has now been reached when suggestive correlations are appearing between the sterols, the nucleic acids, and the carbohydrate metabolism characteristic of the organizer. Some attractive hypotheses come to mind, which we shall consider in the final chapter, limiting ourselves here to a discussion of the established



facts. These already portend an optimistic view for future investigations in this field, provided they concentrate their work on the living organizer itself, or on extracts of it prepared in such a way as to avoid altering the more unstable constituents of the blastoporal lip.

## CHAPTER XI

# Biochemistry of the Organism during Regeneration

Before completing this study of the chemical reactions which accompany morphogenesis, it is in order to say something about the metabolism of the organism during regeneration, an easy task, because of the close analogy between the chemistry of regeneration and of embryonic development.

Biochemical research on regeneration has been principally on worms, chiefly planarians, and on the limbs and tail of urodeles. In organisms able to regenerate, sectioning of a structure is followed by a brief period in which the *blastema* forms, undifferentiated cells accumulating in the region of the wound and insuring the growth of the regeneration bud. Whether these cells arise from a migration of adjacent cells or whether they come from a dedifferentiation of more complex cells still is a controversial question. Several authors attempt to derive the blastema cells from large cells with embryonic characteristics which exist as a reserve in the organism (totipotent cells). Whatever the case, the blastema soon begins to grow and following this morphogenesis takes place.

The field of physiology of regeneration is dominated by the *axial gradient* theory of Child to which we have already referred several times. According to Child an organism such as a planarian shows a physiological gradient decreasing in a cephalocaudal direction, which would explain the fact that the regeneration of a head is less and less frequent as sections are made more posteriorly. This quantitative gradient may be demonstrated, according to Child, by various methods. For example, the anterior end exhibits a "differential susceptibility" toward a number of chemical agents and gradi-

ents in permeability, in respiratory activity and in oxidation-reduction power have also been detected. Rather than review the long series of investigations by Child and his school on the evidence for these gradients, we will limit ourselves here to a discussion of the most recent publications.

Let us consider *reduction gradients* as an example. They are detected by vitally staining the organism with methylene blue or Janus green and then exposing it to anaerobic conditions. This simple method has been used extensively by Child who has reported that decoloration begins at the anterior extremity and gradually progresses posteriorly, the caudal region often being characterized as a second zone of high reducing power. Figure 119, taken from a

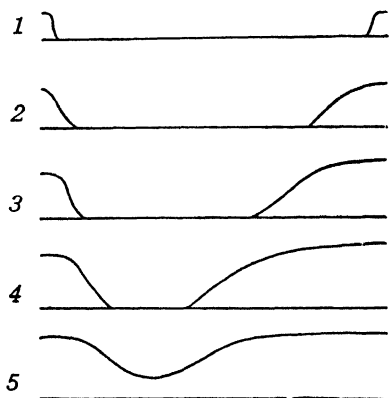


Fig. 119. Reduction gradient in *Tubifex*; the rate of decoloration is indicated diagrammatically (Child and Rulon).

paper by Child and Rulon, shows this phenomenon in the worm, *Tubifex*. Here we see the two opposing gradients in diagrammatic representation.

The conclusions of Child cannot be accepted without reservations, however. Strelin was not able to detect a reduction gradient in a worm resembling *Tubifex*, *Limnodrilus*, because the various organs become unequally colored. He also failed to see such a gradient in the case of *Tubifex* where the excessive toxicity of methylene blue resulted in a rapid cytolysis. Brøndsted carefully studied *Planaria*, one of the organisms frequently used by Child's school, and concluded that the organs combined with methylene blue in too variable quantities to give a homogenous staining. Now it is understandable that the results obtained under such conditions are difficult

to interpret; there does appear to be a clear gradient (Figure 120) but it is complex in nature and bears no relation to the frequency of head regeneration. Brøndsted believes that the differences in reducing power which he has found have nothing in common with the morphogenetic activity of the various regions, but involve, rather, variations in the metabolic rate of each organ taken individually.

But we already know that reducing power is not a measure of the rate of oxidation, and Child considers that the axial gradient is based chiefly on *metabolic rates*. However, it has already been shown that a metabolic gradient in the egg is found only when the cytoplasm is invaded by a yolk gradient as in the amphibians; there are no differences in respiratory rates along the axis of the sea urchin egg or of the chick blastoderm.

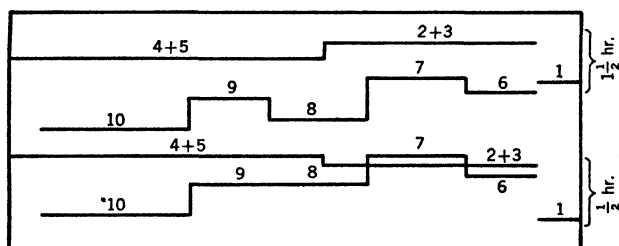


Fig. 120. Reduction gradient in *Planaria*; the figures 1 through 10 correspond to segments more and more posterior (Brøndsted).

One of Child's students, Hyman, has stated that a respiratory gradient is found in various organisms, such as hydroids, sponges and planarians, but the method generally used (Winkler's method for dissolved oxygen) has been subjected to criticism. The manometric measurements made by Shearer have not shown any differences in the oxygen consumption of various regions of the chick embryo and in sections of worms cut along the antero-posterior axis, and measurements of the elimination of  $\text{CO}_2$  carried out by Parker, in sea anemones, *Nereis*, and planaria have led to the same conclusions. In addition, Parker has severely criticized the theory and experiments of Child for, according to him, differential susceptibility would be the result of unequal penetration of toxic substances and is not evidence for the metabolic character of the gradient. Parker is of the opinion that even if metabolic differences exist along the gradient they are

the results, and not the cause, of this gradient. These criticisms of Parker and Shearer are also taken up by J. Needham in his treatise (1931).

Watanabe and Child (1933) undertook to refute these objections. They studied the elimination of  $\text{CO}_2$  (by the colorimetric method used by Parker), the indophenoloxidase reaction, and differential susceptibility in a polyclad worm, *Stylochus*. They noticed, first of all, that cutting the worm caused a change in the metabolism of the fragments. It is only one hour after the operation that one can begin measurements without fear of very aberrant results. With good experimental conditions, if one measures the metabolism of

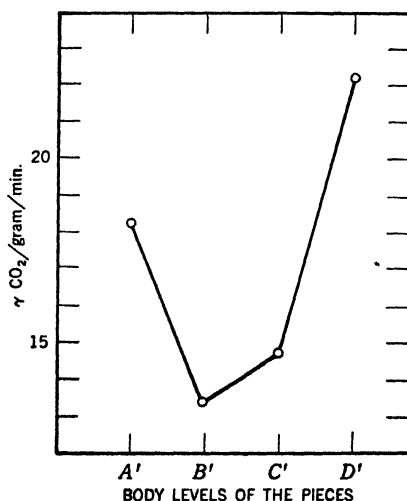


Fig. 121. Gradient in  $\text{CO}_2$  production in *Stylochus* (Watanabe and Child).

fragments 2 to 6 hours after cutting, a U-shaped gradient is found regularly, the anterior and posterior ends releasing more  $\text{CO}_2$  than the middle pieces (Fig. 121). The results of Watanabe and Child confirm those published somewhat earlier by Hyman in *Nereis* (1932).

In addition, Watanabe and Child working on *Stylochus* found gradients in the distribution of indophenoloxidase and in differential susceptibility in the larva as well as in the adult. They maintained the initial point of view of Child and answered point by point the

objections made by Shearer, Parker, and Needham. Although we cannot here enter into the details of this lengthy discussion, it may be said that it is not convincing and we believe that the question will remain open as long as this series of fundamental obstacles is not surmounted. The results of the operation on metabolism, the hemorrhage which follows, the unequal motility of the anterior middle and posterior fragments, all constitute serious sources of error, and it appears that the reality of the axial metabolic gradient in complete organisms cannot be decided on until we possess a technique for carrying out measurements on the intact animal under physiological conditions.

However, it would seem *a priori* that there is little chance for determining a metabolic gradient in forms where the various segments do not contain the same organs. Certain of these organs have a high metabolism which would greatly influence measurements of the part of the individual containing them. For this reason it would be interesting to know if differences exist in the rate of metabolism along the length of an organ common to the whole animal, such as the skin or the digestive tract. An attempt at this was made by Maluf, who compared the oxygen consumption of small fragments of the body wall of the earthworm. He found no differences worthy of consideration but for a somewhat lower metabolism at the anterior end. Thus the conclusions of Maluf are unfavorable to Child's idea, which is hence supported only by work of Child himself and of his students.

It is very probable, then that in the worms, as in embryos, there is no metabolic gradient unless the organism shows some gradual heterogeneity in its structure. In the worm, each organ has its own metabolism which would have its effect on the respiration of the segment where it is found. This is what Brøndsted found for the reduction gradient in planaria. The idea that in an organism as complex as a worm a regular gradient exists seems to have little plausibility, Parker's view of the metabolic gradient as an epiphenomenon appearing more likely than that of Child.

The metabolic gradient theory has recently been tested by von Bertalanffy, who determined with care the growth gradient in planaria and compared his results with those of Child on respiration oxidation-reduction and differential susceptibility. As shown in Figure 122, none of these gradients coincide with any of the others,

and there is no resemblance between the metabolic gradients and growth. One must conclude then that *Planaria* does not show a single axial gradient but that there are probably a bundle of parallel gradients. This conclusion would appear to account for the known facts, and also to be acceptable to Child. Indeed Watanabe and Child concede the existence of parallel gradients and the fact that not all of the toxic agents which they have studied necessarily influence one and the same gradient. However, the main idea of von

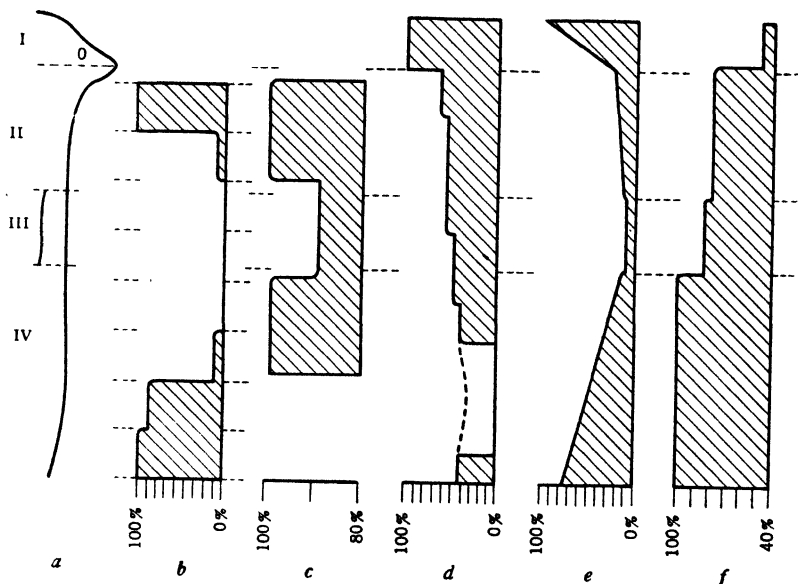


Fig. 122. Comparison of various gradients in *Planaria*: a, diagram of different regions of the animal; b, gradient in the head frequency after cutting into 8 pieces; c, gradient in oxygen consumption; d, gradient in susceptibility to  $H_2O$ ; e, gradient in susceptibility to alcohol; f, growth gradient (von Bertalanffy).

Bertalanffy, that is, the lack of agreement in the growth and metabolic gradients, deals a serious blow to Child's theory, and recent works by von Bertalanffy and collaborators and by Hauschka on the disintegration of planaria in solutions of various quinones and hexenolactone, which combine with  $-SH$  groups, corroborate von Bertalanffy's claims.

We have been concerned up to now with the regional metabolism of the intact organism. What is known of the chemical changes in regenerating tissue?

The values at our disposal concerning *respiration* and *glycolysis* are unfortunately few in number. Okuneff (1933) determined the amount of lactic acid in the limb bud of axolotl two to fifteen days after amputation; he observed a measurable increase in the concentration of lactic acid the values going from 18 to 39 mg. per cent. In addition, there is a lowering of the pH of the blastema, which drops from 6.95 to 6.71. Similar results were published shortly after by Vladimirova. More recently, S. Wolsky compared the oxygen consumption of the regeneration blastema of the tail with that of the normal organ. Working on axolotl, he obtained the following figures:

*Extremity of normal tail* . . . 18.3 mm<sup>3</sup> of oxygen/100 mg. of wet weight.

*Middle or normal tail* . . . 11.1 mm<sup>3</sup> of oxygen/100 mg. of wet weight.

*Regeneration blastema* . . . 32 mm<sup>3</sup> of oxygen/100 mg. of wet weight.

The differences observed have real significance as tested by statistical analysis, and the Hungarian author tends to relate the increase in metabolism in the blastema to its increased water content (82% in place of 75% for the normal tail). A recent paper by Rivkina (1945) has confirmed, by use of Warburg manometers, the production of acids in the regenerate during anaerobiosis and demonstrated large variations in the R.Q. during regeneration. While the R.Q. of the normal limb of axolotl is 1.02, that of the regenerate is much lower (0.59) during the healing phase, 0.57 in the blastema, and 0.72 in the phase of intense differentiation.

We are indebted to Ajsupiet for some measurements of oxygen consumption in hydra after cutting. It was found that respiration decreases during the phase of growth of the blastema and exceeds the normal rate at the time when differentiation sets in. It would appear, moreover, that changes in metabolism are not limited to the blastema and that regeneration has an effect on the oxidation of the whole organism.

Regeneration occurs only in the presence of O<sub>2</sub> and is blocked in the hydroids when the oxygen tension is lowered to 0.3%, at which point the respiration of the organism is lowered to 29% of the normal (Barth). On the other hand an increase in oxygen concentration of the medium favors regeneration, the missing parts reforming faster and being larger in size, while the oxygen consumption increases (Spiegelman and Goldin).



Some interesting experiments by J. A. Miller also show the important role of *oxygen* during regeneration. He placed sections of the stems of a hydroid, *Tubularia*, in a special chamber divided into two compartments; the oxygen tension of each side of the partition could be changed at will. By immersing the proximal end into well aerated sea water and the distal end in water low in oxygen Miller observed a *reversal of polarity*. This effect was also obtained using a thermal gradient. Miller concluded that changing the respiratory metabolism affects the regeneration of the hydranth. It may also be noted that, according to Deotto, regeneration of the missing part in hydroids is accelerated by substances such as pyocyanin and thionin which increase oxidation. This result is in perfect agreement with those of other authors; cyanide, which inhibits respiration, retards regeneration in planaria (Bassina, Finkelstein and Kovarskaja, Rulon) while, conversely, dinitrophenol (Finkelstein and Kovarskaja) and methylene blue (Rulon) increase oxidation and accelerate regeneration.

All these experiments form a coherent whole and thus it is beyond doubt that the maintenance of respiration at its normal level is an important factor in regeneration. However, it would be premature to think that oxidation is the only factor responsible for morphogenesis after cutting. The recent experiments of Goldin and of Goldin and Barth show that oxygen is not a "formative substance" specific for the hydranth, but simply one of the necessary conditions for regeneration. For example, modifications of the pH also can influence polarity. In addition it seems clear that even at a low concentration, oxygen can be utilized for the process of regeneration. In this regard hydroid regeneration is similar to embryonic development.

Among the authors who have tried to influence regeneration by the addition of various substances, the work of Brøndsted on the effects of lithium chloride may be cited. It will be recalled that the  $\text{Li}^+$  ion exercises profound effects on morphogenesis in the sea urchin and in amphibians. In planaria, lithium chloride has no action on head frequency, but a greater percentage of heads with supplementary eyes is obtained. In addition, lithium has a toxic effect on planaria and probably lowers respiration. If this is really so, the gradient in "head frequency" can not be considered as a gradient in oxidations. This deduction is in agreement with the conclusions of von Bertalanffy.

Other investigators have examined the effect of substances influencing cell division, in particular the carcinogens and the thiols, on regeneration. However, these results are very contradictory, for while the carcinogenic hydrocarbons accelerate regeneration in planaria, according to Owen, Weiss and Prince, the opposite is true, according to Tokin, who states that these substances are inhibitory in planaria as well as in the limb buds of axolotl. In addition the carcinogenic substances may inhibit the growth of normal and cancerous cells (Haddow) while chemically similar substances, not carcinogenic, are without action. Colchicine completely inhibits the regeneration of limbs and tails in urodeles (Thornton; Lehmann, Bernhard, Hadorn and Lüscher). Regarding sulfhydryl groups, F. S. and D. W. Hammett have noticed an acceleration of regeneration in the case of the crabs claw. Similarly, Owen, Weiss and Prince, as well as Coldwater, have also obtained favorable results in planaria. On the contrary, Morgulis and Green have had negative results with thiocresol, thiophenol, thioglycolic acid, and cysteine on the regeneration of a polychaete, *Podarke*. Perhaps some of this disagreement rests in the fact that mitosis plays a very variable role in regeneration.

The idea that  $-SH$  groups participate during growth and regeneration in the hydroids receives support from cytochemical observations carried out by means of the nitroprusside reaction. Chapman has reported an exceptionally intense reaction in the regions where growth is most active in *Obelia* (Fig. 123) and this

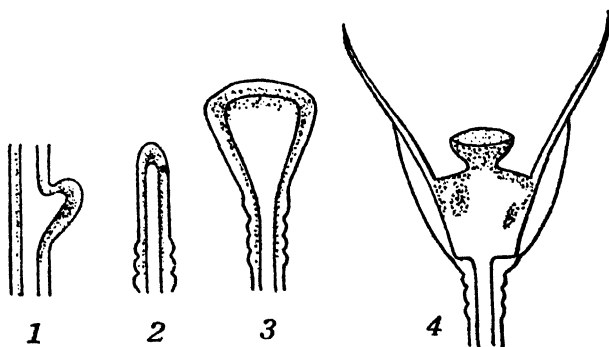


Fig. 123. Localization of  $-SH$  group in the hydroid *Obelia* (Chapman).

fact was confirmed by Hammett and Chapman who noted further an accumulation of tyrosine at the regions of especially marked differ-

entiation; tryptophan and polypeptides, on the contrary, do not show any selective localization. The existence of gradients in the distribution of —SH groups in the hydroid, *Corymorpha*, had been reported earlier by Child and Hyman in 1926. Similar observations were made on the earthworm by Perkins who determined quantitatively the amount of glutathione in various segments.

Hammett and his collaborators have devoted a long series of investigations to the action of the derivatives of nucleic acids and of certain amino acids on the growth and regeneration of *Obelia*. The results are too complex to be analyzed here, so we shall summarize them by saying that xanthine tends to initiate growth, while proliferation is accelerated by hypoxanthine, xanthine, and hydroxyproline; adenine, xanthine, and allantoin stimulate organization, while cytosine, hydroxyproline, and aspartic acid favor differentiation.

The beneficial effect of certain amino acids is also shown by some work of Lecamp. Studying the effects of various amino acids taken singly and in mixtures Lecamp was led to attribute particular significance to *arginine* and *histidine*. These two basic amino acids, together with cysteine and tryptophan, form an excellent medium for regeneration in planaria and in *Triton*. Arginine also favors regeneration of epithelium according to Tarantino and Pasquinelli. These observations can be related to the ideas of Edlbacher, who assigns an important role to arginine and histidine in the growth of tumors and embryos, and they also agree with the results of Caspersson and Thorell which demonstrate that the amount of basic amino acids in cells diminishes when growth is retarded in the chick embryo. One may thus ask whether arginine and histidine do not serve for the synthesis of purines for nucleic acids; this is a hypothesis often advanced but never formally demonstrated.

Mention of these studies suggests a brief review of what is known concerning the *metabolism of proteins* in the regeneration blastema. This problem attracted the attention of Okuneff and Orekhovitch to whom we owe several interesting papers on the subject. They studied chiefly the proteases of the limb bud of axolotl and the natural activators of these enzymes, such as glutathione and ascorbic acid.

Okuneff showed in 1932 that the  $rH_2$  of an amputated limb became reduced, the operation lowering the  $rH_2$  from 22 to 20 where it is maintained for some time. Return to the normal value does

not take place until regeneration is nearly complete. The Russian author attributes this decrease of the redox potential to an increase in the amount of reduced glutathione.

This interpretation was confirmed by the analysis of Orekhovitch (1934), who showed that the amount of reduced glutathione goes from 19.6 mg. per cent in the normal tail to 46 mg. per cent in the blastema of 5 to 11 days. Following this increase is a return to normal.\* It seems clear that the amount of glutathione in tissues near the blastema also increases and that they may also be the site of active proteolysis. Orekhovitch and Bromley and Orekhovitch (1937) were soon able to prove this. Cathepsin activity increased in the regenerate and in neighboring regions without being changed in the remainder of the animal. The amount of dipeptidase also sharply increased and finally exceeded the normal by 216%. In addition, Orekhovitch and Sokolova found that the blastema proteins are more easily digested by cathepsin than those of intact animals.

More recently, the Russian authors have advanced new evidence in favor of their idea. Sokolova has shown that there is a direct relation between the amount of cathepsin of various adult tissues and their powers of regeneration, while Striganova established that limb buds in axolotl use the products of protein catabolism for their regeneration.

These observations have been confirmed by Ryvkina, who showed proteolysis is low when the blastema forms and increases sharply at the time when morphogenesis sets in; it returns to normal during growth of the regenerate. The amount of total glutathione remains constant during regeneration, but the proportion of it in the reduced state is quite high at the beginning of the period of organogenesis of the blastema, decreasing during the third, or growth phase.

It is during the first two phases of regeneration, too, that the highest values for amino nitrogen and for ascorbic acid are found. In short, it would seem that organogenesis in the blastema is characterized by a synthesis of cathepsin, since this enzyme becomes so active when the regenerating part is being reconstituted.

These studies clearly show that the metabolism of the proteins is profoundly changed during regeneration, attaining its peak at the time when organogenesis occurs in the blastema. It would be in-

\* However, it is important to note that Maluf observed a decrease in the amount of glutathione in the blastema of regenerating tail.

interesting to know whether these facts apply to embryonic development as well and to find out whether the amount of cathepsin and reduced glutathione increases at the time when the amphibian larva begins its organogenesis.

Since we know that the *ribonucleoproteins* participate in the synthesis of proteins and that, to all appearances, they play a leading role in morphogenesis in amphibians, it may well be asked if the same holds true in regeneration. This question has been studied intensively by H. Clement and has also been investigated incidentally by P. Brien and by Kedrowski. It has long been known that the "pluripotent" cells, which are poorly differentiated and form a reserve during regeneration or asexual reproduction, are characterized by strong basophilia. For example, the "interstitial" cells of hydra apparently belong to this category and their affinity for basic dyes indicates the presence of ribonucleic acid (Kedrowski). An increase in basophilia has also been reported in the regeneration of *Oligochaetes* (Weitzmann) and in amphibians (Ide-Rozas). In fact, according to Kedrowski there is a synthesis of ribonucleoproteins during regeneration. P. Brien, in the course of extended research on the origin of germ cells and buds in hydroids, has emphasized the constant morphological characteristics of both sexual and asexual reproductive cells, a basophilic cytoplasm, large nucleoli, and intense staining with basic dyes. These characteristics are exhibited when these cells are liberated from the organism and begin to grow, but cytoplasmic and nucleolar basophilia regress at the time when gametogenesis or growth of the bud begins. The use of ribonuclease in these studies by Brien established that the affinity for basic dyes was really due exclusively to ribonucleic acid.

H. Clement studied the distribution of ribonucleoproteins during regeneration in planaria, in the tadpole of the frog, in *Triton*, and in the wound-healing of the skin of the mouse. The same facts noted above are found again, and the picture in planaria is particularly decisive. The animal does not show a gradient in the distribution of ribonucleic acid, a fact that Brachet had already demonstrated by pentose determinations (1941). The various organs have a very variable amount of this acid; skin and muscle contain very little, while the digestive tract is well supplied. The mesoderm has moderate quantities of ribonucleic acid, but the substance accumulates chiefly in the cells which synthesize yolk. When the head is cut

off the blastema is characterized by a very high ribonucleic acid content, and the skin cells during regeneration also show a strong basophilia which is sensitive to ribonuclease. It is difficult to decide whether the blastema forms from the migration of cells rich in ribonucleic acid or whether a synthesis of this acid occurs in the blastema. H. Clement unreservedly supports the latter possibility in the case of the epidermis. It is also to be noted that the amount of thymonucleic acid in the nuclei appears somewhat higher in the blastema, judging from the intensity of the Feulgen reaction. G. Roskin has also observed an accumulation of ribonucleic acid in the regeneration blastema in urodeles with a disappearance of this acid during later organogenesis.

Furthermore, H. Clement has followed cytochemically the distribution of glycogen during regeneration of planaria: no increase in the blastema was found, contrary to the current opinion that considers glycogen to be especially abundant where growth occurs. Castro Rodriguez and N. Pourbaix have reported large amounts of glycogen in the cells which give rise to buds in the asexual reproduction of sponges and tunicates. In any case, the results of H. Clement agree with those of Preto Parvis, who found no correlation between the glycogen content of a cell and its ability for proliferation. Glycogen, according to him, does not disappear during mitosis, contrary to what has generally been believed.

Moyson has recently reported an increase in alkaline phosphatase in the regeneration blastema in both planaria and the frog tadpole. This increase is chiefly in the nuclei and confirms Moog's idea that this enzyme plays a role in the phenomenon of organogenesis.

This brief sketch serves to establish a common biochemical basis for regeneration and embryogenesis. In both cases we see an accumulation of ribonucleic acid, which is an indication of protein synthesis. Also, in both cases, a disappearance of these acids is observed when histological differentiation sets in. In regeneration, as in embryonic development, the active regions show intense carbohydrate metabolism. Synthesis of proteins and utilization of sugars thus are traits common to all morphogenesis.



## CHAPTER XII

# Facts Acquired, Controversial Points, and the Future Outlook

Nearing the end of this review of the field, it seems appropriate to assemble and assess the most interesting results that came to our attention. We shall examine the experimental and biochemical findings and attempt to draw some final conclusions on the nature of the physiochemical processes which are at the basis of embryonic development. In spite of the progress made in the course of the last ten or twelve years it is still not possible to express in precise chemical terms such ideas as gradient, field, determination and potencies which are often used in experimental embryology. This attempt to draw conclusions will then necessarily be imperfect and will occasionally be rather hypothetical.

### 1. Facts Acquired

(a) Without a doubt, chemical embryology has furnished an appreciable contribution to various problems of *cellular physiology*. Indeed, investigations carried out on oöcytes and unfertilized eggs have given a number of exact answers to a series of fundamental questions such as the localization of enzymes in the cell and the respective role of the nucleus and of the cytoplasm in cellular metabolism. In this field, experiments have shown that the classic hypothesis, stating that the nucleus is the center of cellular oxidation, is no longer tenable. We now know that an enucleated fragment of an egg may consume more oxygen than a nucleated half, and it is also clear that the gaseous exchange in the isolated germinal vesicle is low.

It has also been demonstrated that the nucleus, whether it be from a mature egg, an oöcyte, or an ameba is low in enzymes. It is understood, of course, that this conclusion is valid only for those en-



zymes which have been studied up to the present, for it would certainly be dangerous to extend it to other enzymes or to nuclei other than those already analyzed. An important fact has been reported by Duspiva who found that dipeptidase activity increases at the time when yolk formation begins in the frog egg but that this change occurs only in the cytoplasm. In view of this a direct participation of the nucleus in the synthesis of cytoplasmic proteins seems problematical.

It is clear that the study of the distribution of enzymes between the nucleus and the cytoplasm is as yet in a purely descriptive phase and that a thorough quantitative study of large number of nuclear and cytoplasmic enzymes is necessary. It is to be hoped that such a study will bring to light specific differences between the various cellular constituents for if this hope is realized we would be much nearer to an understanding of the physiological roles of the nucleus and the cytoplasm and to an insight into the nature of the interactions between the two. Certain characteristics of this interrelation have already appeared, such as the wealth of —SH groups in the nuclear sap of the oöcyte and the strictly chromosomal localization of thymonucleic acid. The relations between the nucleic acids of the chromatin and of the nucleolus to those present in the cytoplasm are also beginning to appear, while the coupling between the cytoplasmic ribonucleoproteins and the synthesis of ordinary and specific proteins is assuming form.

Present indications afford the possibility of interpreting certain observations on the morphogenetic and physiological role of the nucleus. We refer especially to the beautiful research of Hämmerling on the influence of the nucleus on regeneration in the unicellular alga, *Acetabularia*. His experiments demonstrate that the enucleated fragment survives a long time without alterations in its respiration, nutrition, or assimilation. The cytoplasm deprived of its nucleus thus retains its physiological activities intact, but is not able to regenerate completely as is the nucleated fragment. It seems to us that recent chemical findings agree with these results. Since it is the cytoplasm that contains the highest proportion of respiratory and hydrolytic enzymes, it seems plausible that its physiology would suffer little from enucleation. But regeneration implies a synthesis of proteins controlled by the cytoplasmic nucleoproteins which in turn are dependent upon the nucleic acids in the nucleus, as the ex-

cellent research of Caspersson and Schultz informs us. Thus it is easy to understand why synthesis of proteins stops quickly in enucleated fragments and why regeneration does not take place. In addition, regeneration is accompanied by a synthesis of ribonucleic acid, preceding the synthesis of proteins (H. Clement). It seems clear that the same is true in plants, judging from observations made in Brachet's laboratory. The role of the nucleus in regeneration becomes more intelligible if it is conceived of as controlling the synthesis of ribonucleoproteins in the cytoplasm which in turn control in an indirect way the formation of proteins necessary for the reconstitution of the missing part.

The way in which the nucleus enters into the synthesis of proteins naturally is something of a puzzle. We are tempted to think that this synthesis pertains to the cytoplasmic nucleoprotein granules which Brachet and Jeener have shown to be present in all cells and which possess the requisite properties for the formation of proteins: the presence of ribonucleic acid, of proteases, of ATPase and of respiratory enzymes. It is probable that the chemical constitution of these granules, and perhaps also their number, is regulated by the nucleus. Indeed, we know that the chromosomal formula modifies the amount of ribonucleic acid in the cytoplasm, that is, the number of granules or their ribonucleic acid content. It is clear that this is a valuable line of attack which should be exploited more fully in the future.

Histochemical research on the oöcyte and the egg has established the relative roles of the hyaloplasm and the cell inclusions in cellular metabolism. The results show that the important respiratory enzymes, the concentration of which is determined by the rate of oxidation, are bound to particles which may be displaced by centrifugation. There is perfect agreement on this point between the investigators who have worked on the centrifuged egg or cell and those who have studied extracts of organs. Homogenates contain fine granules which are the sole site of cytochrome oxidase and succinic dehydrogenase. The results in the case of hydrolases are no less significant, since these enzymes are localized to a large extent in the hyaloplasm. Their distribution is not changed by centrifugation and in extracts of organs they are found in large part in the supernatant liquid. However, we must not forget that amylase in the

ameba is chiefly bound to mitochondria, a fact which should warn against premature generalizations. The ideas of Warburg on the role of cellular structure in oxidation are completely confirmed by recent research and probably synthesis of proteins occurs in the region of these same structures.

(b) We will not discuss further investigations of the chemical mechanism of *sex determination*. The main points are that the sexes are determined by substances which are very similar and that the chemical findings are in complete agreement with the biological analyses. The "relative sexuality" theory of Hartmann, for example, is explained formally by the research of Moewus and Kuhn. It is satisfying to witness this fruitful collaboration between biochemists and biologists.

(c) The intimate mechanism of *fertilization* has also become more understandable due to the impetus of chemical embryology. Recent work on the nature of the gamones has revived the interest first stimulated by the discovery of fertilizin. The role of sperm and egg secretions has become clarified and the protein nature of these substances seems almost certain. In addition, chemical analyses lead to a better understanding of the changes which activation brings about. Authentic information has led to valuable discussion of the different theories of fertilization, from which emerges the idea that the unfertilized egg is a cell with an abnormal metabolism. The sea urchin egg, for example, is subjected to a progressive poisoning which slowly lowers oxidation during maturation (Lindahl and Holter). Fertilization restores the metabolic rate to normal while stimulating a hydrolysis of the carbohydrates and proteins. This phase, corresponding to Loeb's cytolysis, is followed by a period of resynthesis (Örström). Finally, the importance of calcium for maturation and for activation is better understood now that we know that bound calcium is liberated at this time. All of these changes are coupled with each other so that one is able to construct a coherent story of the chemical results of fertilization, at least in the sea urchin.

(d) Our knowledge of the chemistry of *cell division* in the segmenting egg has also progressed. The reality of cyclic variations in oxygen consumption remains doubtful, and certainly their amplitude must be very small. In addition, mitosis may often occur in the absence of oxygen. Moreover, it is probable that the anaerobic metabolism of the egg undergoes regular changes, Runnström's ob-

servations on the rhythmic formation of an acid arguing in favor of such changes.

There are also strong indications that —SH groups participate during cell division as a result of which there are cyclic variations in carbohydrate catabolism. Rapkine's hypothesis of a reversible denaturation of the proteins in certain phases of mitosis deserves special attention since it accounts, in a very convenient way, for the changes in viscosity and the morphological structures which appear during division. Modern ideas about the structure of fibrous and globular proteins may thus well explain the structure and function of the achromatic figure.

In addition our knowledge of the localization and role of the nucleic acids during mitosis has also been notably extended, x-ray examination of the structure of nucleoproteins helping to clarify to some extent the changes in form which the chromosomes undergo during mitosis and giving some idea as to the cause of the active migration of these structures toward the centrosomes. It has furthermore been well established that the reduplication of the chromosomes is accompanied by a synthesis of thymonucleic acid, apparently at the expense of cytoplasmic and nucleolar ribonucleic acid. The distinction between partial and total synthesis of thymonucleic acid in eggs is no longer valid, since we now know that the ribonucleic acid of the cytoplasm has two different functions, at once forming a reserve which is used when thymonucleic acid is manufactured by the chromosomes and taking part in the synthesis of proteins. In view of this it would be expected that the amount of ribonucleoproteins would increase every time that proteins are synthesized. There are thus two opposing processes in the egg, utilization of the ribonucleoproteins during mitosis and synthesis when proteins are built up. Thus, in the sea urchin egg where little growth occurs the quantity of cytoplasmic ribonucleoproteins decreases during development in relation to the synthesis of thymonucleic acid, but when the digestive tract becomes active and secretes enzymes of a protein nature there is an accumulation of ribonucleic acid. Conversely, in the chick embryo the blastoderm grows continuously and there is thus a synthesis of proteins and an increase in the ribonucleoproteins, only dividing cells showing a low content of this acid.

The arguments for the functioning of ribonucleic acid during mitosis in the developing egg rests on cytochemical evidence and on

chemical analysis. The work of Caspersson and Schultz has confirmed the direct relationship between the two types of nucleic acids, and we know, in addition, that the formation of the chromosomes is accompanied by a decrease and a simplification of their proteins (Caspersson). Thus we see that our knowledge of the chemical rearrangements which the chromosome undergoes during mitosis has made rapid progress.

(e) The increase in oxygen consumption during development is a constant and well demonstrated phenomenon. Respiration attains a maximum at some definite stage and then begins to fall. Variations in the respiratory quotient during embryogenesis are common, with the early stages being characterized by a preponderance of carbohydrate catabolism. The respiratory quotient then tends to decrease reflecting the *succession of energy sources* which J. Needham has so well emphasized. It should be noted, however, that the newly fertilized egg is often characterized by a low respiratory quotient and it is only after several hours that the utilization of sugars becomes apparent.

There are very good reasons for believing that a part of the energy of oxidation serves in morphogenesis. The work of Tyler and of Bodine makes the distinction between the energy of growth and the energy for differentiation plausible. Whether or not growth and differentiation really require a large portion of the energy resulting from respiration remains a point for discussion. It seems, however, that the major part of this energy serves to maintain the structure of the embryo as was suggested earlier by Warburg.

Recent work gives little credit to the idea that oxidation has a real morphogenetic role. The concept of Loeb, considering respiration as a "variable independent of development," is still quite valid. In certain species, segmentation, gastrulation, and primary morphogenesis comprising neural induction, are possible under anaerobic conditions and thus other reactions producing energy and other enzyme systems can substitute, without difficulty, for respiration and the oxidative enzymes. One must therefore concede that, while morphogenesis requires energy for its realization, the embryo may utilize equally well anaerobic reactions or oxidation. At present we have no reason to believe that the respiratory enzymes, cytochrome oxidase, and the cytochromes, for example, are included among the morphogenetic substances. It is this independence of the oxidative

enzymes from embryonic development that makes one hesitate to share Ries' opinion of the morphogenetic value of the respiratory enzymes with a haemin base in the ascidians. This skepticism is justified by the inherent sources of errors in the methods for the detection of phenolases and by the difficulty in the interpretation of results given by these methods, and receives reinforcement from the fact that the blastomeres containing the phenolases do not show a higher oxygen consumption than the others (Holter and Zeuthen).

Nevertheless, the studies of Ries and of his successors are worthwhile since they demonstrate clearly the heterogeneity of mosaic eggs and prove that the parts of the egg essential for development have different chemical constitutions. The comparison between the eggs of ascidians and *Aplysia* on the one hand, and the sea urchin egg on the other, is particularly suggestive, since it justifies a clear distinction between mosaic and regulative eggs and furnishes a chemical basis for it, bringing out sharply the more or less marked heterogeneity of the cytoplasm.

The interest in this group of investigations lies in having pointed out a problem that is now ready for analysis by quantitative methods. The next step will consist in determining the real chemical composition of the substances described by Ries, Lehmann, *et al.* It may reasonably be asked whether these phenolases can not be identified with the granules studied by Brachet and Jeener which bear the nucleoproteins, the oxidative enzymes, and the proteases. If such a hypothesis can be verified it would be interesting to see if these organelles, apparently destined to aid in synthesis of proteins, accumulate at the regions of highest morphogenetic activity. In such an event, analogies between the invertebrates and the vertebrates may be profitably made.

## 2. Controversial Points

### (a) Gradients

Experimental embryology has very frequently appealed to the idea of gradients to account for its results. Among the investigators who believe that they participate directly in morphogenesis are Boveri, Child, Runnström, Hörstadius, Dalcq and Pasteels, Huxley and de Beer, Spemann, Weiss and others.

Opinion is divided on the nature of these embryonic gradients: Child and Huxley and de Beer have emphasized their *metabolic*

character, visualizing them as gradual quantitative differences in the rate of oxygen consumption along an axis. On the other hand, Boveri, Spemann, and Dalcq and Pasteels identify the gradients with a definite distribution of *substances* in the egg cytoplasm. Spemann and Dalcq, who have discussed the role and nature of the gradients in embryology at some length, refuse to admit that the rate of metabolism is the only factor and they doubt that the integrating concept of Child, namely the notion of "dominance," is applicable to the egg. It is a fact that the dead organizer remains an evocator and that the narcotized dorsal lip of the blastopore still induces; also, thermal gradients have not led to clear-cut conclusions and we have seen that the organizer treated with cyanide remains active in *Discoglossus*. It will also be recalled that it is not sufficient to increase metabolism to make the ventral ectoderm transform into a nervous system, and that this effect may be obtained with substances which do not increase oxidations. All these experiments restrict the application of Child's theory. However, we have to consider two distinct questions: Are there "gradients of substances" in Boveri's sense? Are the metabolic gradients of Child real ones?

The most cursory examination of amphibian eggs clearly demonstrates the presence of a vitelline gradient, the size of the yolk platelets increasing in regular fashion from the animal pole towards the vegetal side. Cytochemical techniques allow still further analysis and there is no doubt that a sulfhydryl-ribonucleoprotein gradient exists, counter to the vitelline gradient. The latter gradient becomes very apparent when cytochemical tests for arginine and tyrosine are applied to the egg, for the yolk granules at the vegetal pole give a particularly intense reaction. At gastrulation the dorsal lip of the blastopore is characterized by large amounts of ribonucleoproteins, the primitive animal-vegetal gradient becoming complicated at this time by the appearance of the dorsoventral gradient. Subsequently the gradients are still evident and in the neurula one observes a cephalocaudal gradient in the axial organs combined with a clear dorsoventral gradient in the region of the chordamesoderm. In addition, when organs are differentiating, foci for the synthesis of ribonucleoproteins are detectable, which are very striking in the case of the sense organs. Thus it can not be denied that in the amphibians the sulfhydryl ribonucleoproteins are distributed in gradients paralleling those which experimental embryology has discerned. We

have seen that these same facts hold true somewhat less clearly, in other vertebrates.

The presence of ribonucleoprotein gradients in the invertebrates is much less certain, but the question has not been systematically studied in these forms and a yolk gradient opposed perhaps by a nucleoprotein gradient probably exists in invertebrate eggs rich in yolk such as those of worms, mollusks and ascidians. Spek's observations on bipolar differentiation show some evidence for this and Gersch showed that "basic" colloids which accumulate at the animal pole at maturation are very basophilic after fixation, which almost certainly indicates the presence of ribonucleoproteins. According to Raven's observations the maintenance of this bipolar differentiation is essential to normal morphogenesis and thus one must conclude that the precise distribution of ribonucleoproteins and of yolk is one of the necessary conditions for development. This is a point to which we shall return later.

But, considering the case of the sea urchin egg, where the presence of two "antagonistic" gradients between the animal and vegetal pole is so clearly shown by the research of Runnström and especially by that of Hörstadius, we meet with almost complete failure in an attempt to demonstrate a gradient in the distribution of chemical substances. None of the methods thus far used show such a gradient with any certainty, although it may be that there is a weak "gradient of reduction" along the animal vegetal axis. Lindahl has reported that the micromeres are especially basophilic. Brachet has confirmed this fact and demonstrated that this is due to an increase in ribonucleoproteins. The same conclusion was drawn by Monné working in Runnström's laboratory. This result is interesting because of the great morphogenetic value of the vegetative material which Hörstadius has once claimed to be a true organizer. But, for all this, we are still simply dealing with a local accumulation of the ribonucleoproteins which do not show a true gradient visible under the microscope. Of course one can not *a priori* reject the idea that particular ribonucleoproteins bound to granules, for example, are distributed in gradient fashion, but this supposition is gratuitous at the present time. Without completely negating this idea we must recognize that we still do not know the possible nature of the "substance gradients" in the sea urchin egg. Nevertheless, certain facts suggest that the ribonucleoproteins play a role in the morphogenesis



of the sea urchin just as in the amphibians. In particular, E. B. Harvey has obtained normal development of ultracentrifuged fragments which are completely free of visible inclusions and these fragments are especially rich in ribonucleic acid as shown by Harvey and Lavin, as well as by Runnström and Monné.

Proceeding to the metabolic gradients, as Child thinks of them, the evidence at our disposal pertains to eggs as well as to complex organisms such as worms. We shall not consider the case of the latter, since the results are too contradictory.

In regard to the egg we must distinguish between forms with and without an easily recognizable yolk gradient. In the sea urchin, where this gradient is slight or lacking, the measurements of Lindahl and Holter establish clearly that there is no difference in the intensity of respiration between the two poles. If there is a gradient in metabolism it seems only to be a U-shaped one, where oxidations are high at the poles and low at the equator. However, according to Child himself, there is only a simple gradient revealed by the decreasing reducing power along the animal-vegetal axis. This hypothesis, then, seems to be vitiated by the experiments of the Scandinavian authors. As to the assumption that metabolism is weaker at the equator as compared with that at the two poles, it is clear that this is in agreement with the idea that each pole is the point of departure of opposing gradients. We have the impression, however, that it would not stand up under experimental test, which, however, it deserves. One can not be sure as yet that the metabolism at the two poles is *qualitatively* different, as Lindahl suggests, and determinations of the respiratory quotients of both hemispheres are necessary to prove the point. Such measurements are extremely delicate but the difficulties are not unsurmountable. Only the R.Q.'s would put us in a position to take a definite stand for or against Lindahl's hypothesis.

It is well to include here a reference to the measurements of Philips on the oxygen consumption of the various parts of a young chick embryo, since in this case the yolk gradient is negligible. The determinations were made at a time when primary morphogenesis is in operation, when the metabolic gradients should be acting, but the author concludes that there are no differences in the rate of oxidation from one region to another. These findings deal a serious, if not decisive, blow to Child's theory as applied to embryonic develop-

ment, since, if metabolic gradients are present along the axis of the chick embryo they can only be *qualitative* in nature. However, Child emphasizes the purely quantitative character of metabolic gradients.\* In the case of the chick only "substance" gradients such as those of ribonucleoproteins and (according to Jacobson) of lipids, have been distinguished.

We have discussed the metabolism of the various parts of the amphibian egg in enough detail so as to simply touch upon this subject here. The yolk gradient is very sharp and it is not surprising that it is coupled with an animal-vegetal metabolic gradient. We have also seen that the medullary plate consumes more oxygen than the lower part of the neurula, this being good evidence in favor of a dorsoventral respiratory gradient at this stage. In addition, the head end of the advanced neurula respire more actively than the posterior parts and thus a cephalocaudal oxidation gradient is present at this time. During gastrulation the dorsal lip of the blastopore, in spite of its high yolk content, shows a high respiration but not as high as the maximum respiration in the embryo. The presumptive nervous system, very low in yolk, respire at a greater rate than the organizer. It will be recalled that the rate of metabolism of various regions of the egg is parallel with the ribonucleoprotein content and it can only be concluded, therefore, that the respiratory enzymes associated with ribonucleic acid in the ribonucleoprotein granules limit the rate of oxidations.

Such an assumption agrees perfectly with the idea expressed by J. Needham in his latest book (1942) that the presence of an iron-catalyzed respiration based on cytochrome oxidase and cytochromes appears to be necessary for all morphogenesis. It also agrees well with Potter's results establishing that the different enzymes functionally coupled with cytochrome are ribonucleoproteins (see Chapter VI).

Most of the investigators who have studied the metabolism of fragments of the amphibian egg have drawn conclusions unfavorable to Child's theory, but we do not believe that this attitude is justifiable, since Child conceives of the organizer as a secondary center of metabolism imposed on the primary animal-vegetal gradient.

\* In a recent article Child (1946) has stated that metabolic gradients are quantitative only at the beginning of development and later they become qualitative in nature. In this form Child's theory is certainly more acceptable.

Thus oxidation in this region increases little by little and it is only at the time when induction occurs at the end of gastrulation that the organizer is the high point of respiratory exchanges. However, there is one aspect of this question where the facts clearly contradict Child, since we know now that the metabolism of the organizer is *qualitatively* different from that present in ventral regions. The metabolic gradients in the amphibian gastrula can not therefore be purely quantitative as Child conceives them.\*

We do not know as yet whether the carbohydrate metabolism that is so marked in the organizer has any real morphogenetic significance. The fact that dinitrocresol intensifies glycolysis of ectodermal fragments without provoking neural differentiation leads one to believe that the catabolism of sugars is not of itself sufficient to release the inductive substance.

By way of conclusion we may say that, at present, a number of eggs show gradients in the distribution of certain substances, notably the ribonucleoproteins. These gradients coincide with those predicated by other types of experimentation, since their maxima are always found in regions where morphogenesis is particularly active and they are most apparent in eggs where the yolk gradient is clearest. Because of the inertness of the yolk with respect to metabolism, these gradients are accompanied by respiratory gradients, at least in amphibian eggs. The latter gradients are certainly complex because qualitative differences are superimposed on quantitative differences. The association of a component of the ribonucleoproteins with oxidative enzymes makes it easy to understand the identity of the ribonucleoprotein and the respiratory gradients. These conclusions suggest that the rate of synthesis of proteins is unequal in the various regions of the egg and that it varies according to the intensity of the gradients.

*(b) Nature of Inducing Substance and Physical Basis of Morphogenetic Movements*

No one can reasonably doubt the idea that the organizer exerts its effects through the mediation of a chemical substance. This is the opinion held by all the investigators who have had personal experience with the problem, and if there are any persons still unconvinced we point out that Waddington has calculated that the prob-

\* See preceding footnote.

ability that the differences he observed between coagulated egg white and the same substance impregnated with carcinogenic hydrocarbons are due to chance is 1 in 3,000,000.

Among recent investigators, Moricard and Gothié alone propose a different interpretation. They think, briefly, that the induction they obtained after microinjection of a droplet of sodium oleate into the gastrula may result from a change in surface tension. However, this explanation may be objected to on the grounds that sodium oleate is an excellent substance to break down lipoprotein complexes in trace amounts (Peters and Wakelin) and thus has the requisite properties for easily liberating the evocator from an inactive complex. It will also be noted that the substances injected by Moricard and Gothié were effective only when they were cytolyzing agents and that Gothié observed no effect with a very surface active detergent while sodium butyrate, which is only mildly surface active, may produce inductions.

It must be confessed at once that the chemical nature of the substance responsible for induction remains unknown. Although the sterols are active in low concentration they also stimulate abnormal mitoses in very low concentrations. The theory of a non-specific stimulus by acid does not appear valid in all cases and if dead tissues owe their inductive power to their nucleic acids there is still nothing to prove that these acids are responsible for normal induction. Certainly the behavior of the ribonucleoproteins during gastrulation and neurulation leads one to assume that these substances play a role in neural induction in the living embryo, but one cannot decide whether these nucleic acids or their derivatives are identical with the inducing substance or whether they are only intermediates in a chain of reactions leading to the formation of a neural plate.

There are some experimental results that deserve a fuller discussion. First of all, the fact that the dead ectoderm becomes an inductor is still not explained satisfactorily. The hypothesis of a liberation of an inducing substance at the expense of an inactive complex at the time of killing of the fragment is attractive, but it can not be considered as demonstrated and it is necessary to see whether other explanations are not possible. It seems to us that not enough emphasis has been placed on the fact that only living fragments of the gastrula are perfectly incorporated into the tissues of the host.

For example, a layer of presumptive epidermis is subjected to the influence of the host when it is grafted into a new site of the gastrula. If we transplant it into the region of the dorsal lip of the blastopore, as done by Raven, it acquires inductor ability and one might suppose that under these conditions its carbohydrate metabolism would increase and its nucleoproteins would be modified since both of these changes may lead to the synthesis of the inductor substance. On the other hand, when such a fragment is grafted into the ventral region of the embryo where it comes in contact with cells low in nucleoproteins and with a low metabolism there is nothing to stimulate its activity and we can see why it will not induce. What takes place when this same fragment is killed and then transplanted? It is no longer assimilated by the host and as inert material is attacked by enzymes just as any other grafted tissue. Nucleases will hydrolyze the nucleic acids, resulting in an accumulation of nucleotides which act as evocators between the graft and the ectoderm of the host. If, for the sake of argument, we admit that the inductor is a nucleotide originating from ribonucleic acid, we arrive at the following explanation. The living ectodermal fragment transplanted into the organizer region undergoes, like the dorsal lip itself, a hydrolysis of its nucleic acid; when it is grafted ventrally, however, this hydrolysis does not take place because in the living state neither the graft nor the surrounding cells are capable of reacting in this manner. However, if a dead fragment is implanted it injures the neighboring cells which release nucleases which then hydrolyze the nucleic acids of the graft. It is possible to subject this hypothesis to experimental verification.

This interpretation differs from that of Needham and Waddington who assume that the evocator is liberated from a complex in the ectoderm by killing. Brachet believes that the dead ectoderm is attacked by the surrounding cells and that out of this reaction evocator substances, which are not necessarily identical with those of the living organizer, are produced. This explanation receives support from well known facts such as the hydrolysis of the nucleic acids of the graft by ribonuclease of the host and the high evocator activity of certain products of this hydrolysis.

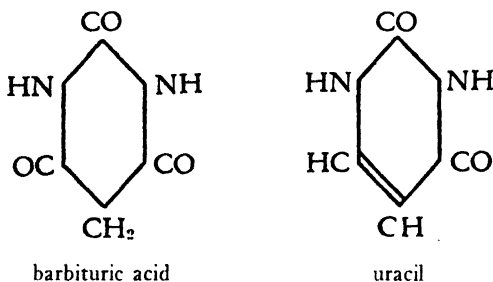
But why does the dead organizer retain only its evocator property and lose its power of individuation? This problem was discussed in a very penetrating way by Waddington (1938), whose ex-

planation of the specific regional induction by the organizer stemming from regional organization was as follows: the organizer contains a "basic evocator" the action of which is changed by "modulators"; individuation would be possible if the evocator were distributed in the form of an axial gradient, but it may also be that regional specificity depends on specific spatial relationships such as the formation of partially oriented molecules, the formation of liquid crystals, specific surface properties, etc. These explanations appeared more plausible to Waddington than the hypothesis of specific substances each inducing a different part of the nervous system. It should be pointed out here that if the normal structure of the neural tube depends on a gradient distribution of the evocator, it is easy to see that dead tissues would be only simple evocators since the gradient would be lacking and they would induce atypical nervous systems lacking regional differentiation. One may also picture the proteins of the organizer as the agents producing individuation (Barth), whose denaturation would cause all specificity of the organizer to disappear. Brachet has pointed out that we may also assume that the active agent is a conjugated protein where the prosthetic group is the evocator and the protein is the modulator. These suppositions find a certain amount of support in the observations by Chuang, who demonstrated that a brief heating, which would denature proteins, changes the specificity of heterogenetic inductors.

Since we now find ourselves in the realm of assumptions, let us take the liberty of formulating some hypotheses as to the chemical mechanism of induction in the living embryo. The future will perhaps demonstrate their inadequacy, but since they are subject to experimental verification they merit consideration. As a point of departure let us admit that the ribonucleoproteins participate in normal induction. This assumption is based on cytochemical observations, confirmed by pentose determinations of various regions of the egg, which show that the amount of ribonucleic acid of the organizer decreases during invagination while that of the presumptive nervous system increases. At the end of gastrulation we observe a cephalocaudal gradient in the neural ectoderm and an opposed gradient in the presumptive notochord (Fig. 90). Furthermore we know that ribonucleoproteins accumulate at the interface between the inductor and the presumptive nervous system (Fig. 91). Finally, it is a fact that nucleic acids and certain nucleotides are evocators and that

the inducing power of dead organs or of nucleoproteins is lowered when they are deprived of their nucleic acid.

The above hypothesis gains support from certain other facts. Woods has established that the multiplication of certain bacteria is inhibited by the addition of barbituric acid, which antagonizes uracil due to a similarity in molecular structure. Uracil is indispensable to the growth of these bacteria probably because it is necessary for the synthesis of ribonucleic acid.



Now, Brachet has shown (1945) that the development of frog gastrulae treated with barbituric acid exhibits serious abnormalities. The morphogenesis of the head is rudimentary and neural induction is lacking in the posterior half of the embryo which remains in a gastrular condition, while at the same time the synthesis of nucleic acids is inhibited considerably. It is to be noted that uracil exerts hardly any effect on development. These experiments illustrate in an indirect manner the direct coupling between the synthesis of nucleoproteins and induction.

Strong inhibition of neural induction is also obtained by treating the gastrula of frog or axolotl with benziminazole, which competes with adenine (which it resembles chemically) in bacteria and in yeast, according to Woolley.

It is clear that benziminazole, like barbituric acid, tends to slow the synthesis of nucleic acids in treated embryos as shown by the cytochemical examination of treated embryos. However, we can not say in the case of embryos that the primary action of this substance is really specific, since it is not possible for the embryologist to determine whether development will proceed after addition of adenine or uracil as in the case of the bacteriological studies. In fact, it is only necessary to place the treated eggs in water to restore develop-

ment. In addition, it must not be forgotten that benziminazole and barbituric acid are narcotics and inhibit cytoplasmic division of segmenting eggs possibly by modifying the viscosity of the cell.

We may further cite in favor of a coupling between synthesis of nucleoproteins and induction a number of studies showing a high frequency of degenerative phenomena, particularly pycnosis in cells, in regions of the embryo where development is most active (Vogt, Stockenberg, Glücksmann, Wigglesworth, *et al.*). As we have noted (1940) these cells have a particularly high ribonucleoprotein content in the cytoplasm. Again we see a striking correlation between the amount of ribonucleoproteins and the intensity of morphogenesis.

Finally, Brachet has obtained almost complete inhibition of neural induction by treating gastrulae with acriflavine, which combines with the nucleic acids and nucleotides (McIlwain). Mesodermal structures may form normally although the nervous system is practically absent. In certain favorable cases, one can restore development by adding nucleotides to the blocked embryos, making it apparent that in these cases the block to morphogenesis was brought about by a disturbance in the metabolism of the nucleic acids or their derivatives.

It will be remembered that this same correlation between ribonucleoproteins and induction appears again in cytolyzing embryos, where the cells become richer in ribonucleic acid which later disappears, and in centrifuged eggs. When the egg is centrifuged before cleavage, acephalic embryos are obtained, which fact is explained by the accumulation of ribonucleoproteins at the animal pole and their presence in smaller quantities in the organizer (Pasteels). On the contrary, centrifugation in the gastrula stage results in double embryos probably because the ventral ectoderm comes in contact with cells rich in nucleoproteins; the secondary nervous system of these embryos is particularly rich in ribonucleic acid (Pasteels and Brachet) and the same situation holds for all inductions produced by an implant of an organizer or an evocator.

To return to our working hypothesis, during invagination the ribonucleoproteins are broken down to nucleotides, and some of these then transform into activators of carbohydrate catabolism, such as coenzymes or adenosine triphosphate. In this way the metabolism of sugars characteristic of the dorsal lip of the blastopore is initiated. The remainder of the nucleotides diffuses toward the presumptive



nervous system and contributes to the synthesis of new ribonucleoproteins. Thus we may explain the two opposing gradients in ribonucleoproteins at the end of gastrulation and the fact that they are found in such abundance at the border between the roof of the archenteron and the neural ectoderm. Intense carbohydrate metabolism in the future dorsal half of the egg should favor a synthesis of ribonucleoproteins in the presumptive nervous system. Indeed, Brachet and Jeener showed that zymonucleic acid is synthesized in yeast only when the carbohydrate metabolism is intact. Also, we know that during the oxidative decarboxylation of sugars ribosephosphoric acid is formed and this enters into the constitution of ribonucleic acid (Dickens, Lindberg). Naturally it may be that other substances, the sterols for example, might take part in the hydrolytic splitting of nucleic acid in the organizer and in its synthesis in the presumptive nervous system.

We know now that there is a direct relationship between ribonucleic acid and protein synthesis and thus there are grounds for thinking of a formation of new specific proteins in the neural ectoderm at the end of gastrulation. This is accomplished at the expense of the yolk which rapidly disappears in the nervous system. These new proteins in the neural plate may be thought of as having elongate molecules, not a completely theoretical assumption since Lawrence, J. Needham and Shen have recently demonstrated fibrous proteins similar to myosin in the neurula, while Hobson has observed birefringent structures in the neural plate of the chick even after fat extraction, chiefly in the cell walls which are rich in nucleoproteins. However, it must be pointed out that the fibrous proteins studied by Lawrence, Needham and their collaborators were isolated at a stage where the embryo contains a large number of nuclei and thus it is possible that the extracts prepared by the English authors were contaminated with thymonucleohistone which has very elongate molecules. In fact, Rebuffat and Brachet have found that the fractions, which were studied by Lawrence *et al.*, and which contained fibrous proteins as shown by viscosimetric measurements, also contained an appreciable proportion of thymonucleic acid. The presence of fibrous proteins in the cytoplasm of amphibian embryos does not appear to be well established. If the proteins formed in the neural plate are really analogous to myosin of muscle, it is conceivable that the contraction of their molecules would modify the shape of

cells and in this way lead to the closure of the nervous system. The idea that the movements of gastrulation and of neurulation result from deformations in molecular structure of proteins was suggested in 1937 by Harrison. Among the reactions which would lead to changes in the form of protein molecules are the condensations of the —SH groups into —SS—. In this way we could explain how the blocking of —SH groups prevents the closure of the neural tube without affecting the inducing power of the organizer (Fig. 105).

This assumption is the more reasonable since Godeaux has recently shown that myosin fibers fail to contract in the presence of adenosine triphosphate and  $Mg^{++}$  ions when the —SH groups are previously blocked. It would be interesting to repeat these experiments on the fibrous proteins of the neurula.

Some valuable information on the probable localization of fibrous proteins which may be responsible for morphogenetic movements has been gathered. In the gastrula the cells of the dorsal lip of the blastopore are pear-shaped, and Waddington has found that the thin prolongations are slightly birefringent and thus should contain elongate molecules. Another indication is the arrangement of the yolk platelets in parallel fashion in these prolongations. These platelets show no orderly arrangement in the cells of the neural plate. Waddington measured the surface tension of cells from different regions of the gastrula and the neurula and found great differences. The resistance of cells placed in contact with the interface between water and an organic liquid is highest for the neural cells and lowest for the endoderm. These observations are favorable to the hypothesis of a cortical localization of oriented fibrous molecules.

Waddington's hypothesis explaining morphogenetic movements by the action of oriented fibrous molecules is not the only possible one, as has been pointed out by F. O. Schmitt. This author first of all rejects the older theory of Glaser which attributed the formation of the neural plate to a localized hydration. The measurements by Hamburger, Brown, and Schmitt on the density of various parts of the egg certainly exclude the possibility that the neural plate has an especially high water content. The fibrillar theory of Waddington, and that of an oriented cytoskeleton upheld by Harrison and Astbury, still lack a really experimental basis. Therefore, Schmitt has proposed a new and ingenious hypothesis which holds that any sub-

stance that would increase cohesive forces, and, as a result, the adherence, of the surface of the epidermis would be an evocator. Indeed this change in cohesive forces would tend to transform the cuboidal cells of the ectoderm into columnar cells characteristic of the medullary plate. The following experiment serves to make the theory more concrete. We know that red blood cells have a membrane in which acid groups are preponderant because of cephalin. If one adds to a suspension of red blood cells a trace of a basic protein, a histone for example, it tends to form a monomolecular layer between two bimolecular layers of cephalin. As a result the cells become spherical and adhere to each other forming a sort of morula. The cohesive forces between the cell membranes are increased in the presence of calcium, decreased by lithium.

By analogy with these experiments with red blood cells, Schmitt thinks that all substances decreasing the solvation of the membrane will increase intercellular adhesion. The position of a monomolecular layer of histone in a membrane rich in acid groups will act as a zipper. Such an explanation accounts for the organizer effect not being stopped by cell membranes. Holtfreter's research has shown that the surface coat forms a true syncytial covering. Schmitt's theory also explains how chemically different substances may function as an evocator and accounts for the inhibitory effect of lithium. But, like the other explanations proposed by various authors, it can not be considered as demonstrated experimentally at present.

The observations and experiments of Holtfreter, which we cannot describe in detail here, demonstrate the importance of the surface coat of the egg. Probably it is a network of fibrous molecules appearing in the unfertilized egg which in an alkaline or calcium free medium tends to disintegrate and the cells separate. This surface coat is responsible for the regulation of the osmotic properties of the egg and its elasticity permits morphogenetic movements. The latter would result chiefly from the expansion of the cells due, perhaps, to an unfolding of the cyclic protein molecules which are subject to a reversible surface denaturation. Holtfreter's ideas are very similar to those of Rapkine (personal communication) and of Schmitt and they are in agreement with recent studies on the mechanics of gastrulation and neurulation by Gillette, Burt, and Nicholas. As to invagination, it would result, according to Holtfreter, from a lowered surface tension of the ectodermal cells as compared with underlying

cells. The alkalinity of the blastocoel (Buytendyk and Woerdeman, Gregg and Ballentine) may be one of the factors influencing the surface tension of the cells which form its lining.

It can be seen that the important studies of Holtfreter and Waddington open new horizons, pointing the way to fruitful research.

These speculations on the role of fibrous proteins in morphogenesis, so attractive in themselves, find some justification in the comparison which can be made between the nervous system and muscle. In the latter, as has been demonstrated by a variety of workers (Engelhardt and Ljubimova, J. Needham, Shen, D. M. Needham and Lawrence, Szent-Györgyi and collaborators, D. M. Needham, Bailey, Caspersson and Thorell, Dainty, Kleinzeller, Lawrence, Miall and Needham, etc.), there is a direct relation between myosin and the hydrolysis of a nucleotide, adenosine triphosphate, which furnishes the energy and participates in the breakdown of carbohydrates. One may envisage the possibility that in the neurula, too, the metabolism of the sugars, nucleic acids and their derivatives, and the morphogenetic movements may be coupled to some extent. Perhaps this coupling occurs earlier at the time of invagination of the organizer during gastrulation. It is clear that if it could be shown that in the embryo events are similar to those in muscle, the intimate mechanism of morphogenesis would soon become clarified.

The first step in this direction has just been taken by Brachet and Chantrenne who compared the amount of adenosine triphosphate in dorsal and ventral halves of the embryo in the gastrula and neurula stage with the following results:

Adenosine Triphosphate in  $\gamma$ /mg. N

Early Gastrula	Pentose	P
Presumptive neural plate and notochord .....	25.1	24.6
Remainder of the embryo .....	13.4	12.6
Early Neurula		
Nervous system and notochord .....	37.5	39.6
Remainder of the embryo .....	11.4	11.4

It is seen that gastrulation and neurulation are accompanied by a synthesis of adenosine triphosphate which is lacking in morphogenetically inactive regions. This fact suggests that this compound,

which may act as an evocator (Fischer and Wehmeier), plays a role in the process of induction. In addition, preliminary trials have shown the presence of adenosine triphosphatase in the gastrula, although the localization of this enzyme has not yet been studied, and the presence of adenosine triphosphate has also been demonstrated by Barth and Jaeger, who have shown that this compound is hydrolyzed reversibly under anaerobic conditions.

However adenosine triphosphate is not the only nucleotide which is synthesized exclusively in the dorsal half of the egg during gastrulation, for both ribonucleic acid and thymonucleic acid behave this way, as shown from some analyses by Brachet and Chantrenne.

Early Gastrula	Ribonucleic acid in $\gamma$ /mg. N	Thymonucleic acid in $\gamma$ /mg. N
Presumptive neural plate and notochord .....	7.6	7.6
Remainder of embryo .....	5.9	8.4
Early Neurula		
Nervous system and notochord .....	10.5	12.7
Remainder of embryo .....	5.7	8.3

Thus we see that both the metabolism of the nucleic acids and their derivatives are stimulated during induction.

Let us briefly conclude the sketch which we have just outlined. Evocation by heterogenetic inductors can be easily understood and in the majority of cases the mechanism might be that just proposed: that the nucleic acids of the graft become hydrolyzed and the nucleotides formed take part in the synthesis of ribonucleoproteins in the adjacent ectoderm, where new proteins are formed and a structure arises resembling a nervous system. The absence of individuation is paralleled by an absence of a gradient in nucleic acid and its derivatives, for it will be remembered that the existence of such gradients in the normal gastrula is clearly shown by cytochemical examination. The inductions of specific organs may be due to the "modulators," the nature of the protein which is combined with the nucleic acids being the determining factor.

There remains to be explained those inductions brought about by agents not containing nucleic acid, such as concentrated acids, methylene blue, sterols, and carcinogenic hydrocarbons. One might

assume that they have a purely cytolytic action on neighboring cells, for during cytolysis a momentary increase in the amount of ribonucleic acid is observed in the cell soon followed by a disappearance of this acid, which then is transformed into mononucleotides which diffuse out of necrotic cells and thus produce evocation. This mechanism must certainly play a role in some cases, especially those of concentrated acids. For the dyes and sterol derivatives we believe another explanation is preferable. It will be recalled that an important fraction of the ribonucleoproteins present in amphibian eggs is bound in "granules" which have their counterparts in all cells. Now, Graffi demonstrated that a carcinogenic hydrocarbon, benzo-pyrene, selectively accumulates in the granules when cells are immersed in a solution of this substance, and Brachet has shown, with Chantrenne, that the isolated granules of various organs adsorb methylene blue intensely. The properties of the particles which have adsorbed a polycyclic hydrocarbon can thus be modified, and we have seen that these granules, to all appearances, are the agents which synthesize proteins. May we not assume that their ability to synthesize proteins is stimulated by the adsorption of a sterol or a dye? This is why we believe it is legitimate to suppose that during normal induction a sterol becomes fixed to the granules and stimulates their function.

Perhaps later research may prove that these granules are the same as the "inactive" complex of the English authors. Up to the present we have never obtained evocations by grafting a mass of non-fixed granules, but many of the embryos so treated have been infected. Granules after fixation by alcohol or heat are, on the contrary, good evocators and the nucleic acid which they contain is rapidly removed by the nucleases of the host. The hypothesis of an identity between granules and the inactive complex gains credibility from Kedrowski's research which indicates that the ribonucleoproteins are liberated from a complex during development. It is clear that nucleic acid is bound to the granules in a labile manner since heat, acids, alkalis, ultraviolet light, salts (Claude and Rothen, Brachet, Jeener and Chantrenne) liberate it quickly. The future will show, sooner or later, how much of the relationship just proposed is valid.

However, we might point out that a similar situation has developed in the case of other cellular organelles, in particular the

lipochondria recently studied by Holtfreter. These granules are composed of lipids surrounded by a protein layer which breaks down when the protein is denatured. During development the lipochondria tend to be replaced by free fat droplets according to Holtfreter. This observation lends credence to Rapkine's hypothesis that a reversible denaturation of the proteins plays a fundamental role in morphogenesis and in particular in the induction of the nervous system.

The idea that the properties of the ribonucleic acid granules become altered when they adsorb a polycyclic hydrocarbon has already been upheld by Graffi in the case of tumors. Indeed he has put forth the hypothesis that the granules, which he compares to mitochondria, may, to some extent, bring about malignant tumors when they have adsorbed a carcinogenic agent. These abnormal mitochondria may mutate to a virus capable of transmitting the tumor. This supposition finds support in the experiments of Selbie and McIntosh whose importance to the experimental study of cancer cannot be overestimated if they can be confirmed. These authors have found that a filtrate of tumors, which had been stimulated by hydrocarbons, is able to transmit the tumor.

This leads us to sketch briefly the parallel between malignant tumors and the organizer. Since this question has been treated in detail by J. Needham (1938) and by Waddington, we will here only emphasize some similarities on a biochemical level. Tumors, like the nervous system of the embryo, may be stimulated by polycyclic hydrocarbons in low doses. In both cases one finds the cells possessing a high carbohydrate metabolism and, furthermore, tumors, like the organizer and the presumptive nervous system, are rich in ribonucleoproteins (Caspersson and Santesson, Mitchell, Biesele, Khouvine and Gregoire, Schneider, *et al.* in the case of cancer). The frequency of tumors following painting of the skin with a carcinogenic agent decreases if one first blocks the —SH groups (Berenblum, Crabtree). We have seen that such treatment also inhibits the induction of the nervous system without hindering the formation of the notochord. As a result, in both cases, the ability of the cells to react to the stimulant ("competence" of Waddington) decreases after blocking the —SH radicals. The role of the —SH groups in cancer and in the induction process must naturally be related to Rapkine's ideas on the denaturation of the proteins in the dividing

cells and in the organizer. It is interesting to note in this regard that Rondoni has recently upheld the idea that cancer results, in the first place, from a denaturation of the proteins.

The analogy between tumors and inductions is shown chiefly in evocations without individuation, as the latter are atypical and chaotic and analogous to teratomas in pathology. Needham calls attention to the curious observation by Witschi that the overripening of amphibian eggs often leads to the formation of double monsters. If the process is carried further, true malignant epitheliomas develop in place of the secondary nervous system. All the transitions between cancer and induction are thus found and some very similar chemical reactions must be at the basis of the two phenomena. Why then are tumors merely chaotic growths devoid of form? The answer probably lies in the fact that organization into a gradient, so characteristic of embryos, is lacking.

It is of interest to see, in view of all this, whether Graffi's concept can be extended to embryonic development. We must assume that induction would be stimulated by a substance able to undergo autosynthesis *in vivo* of the same character as a virus or a gene. This possibility had been envisaged before the research of Graffi, by Dalcq and Pasteels. Here is the way Dalcq summarizes his point of view (1942, page 493 of this paper): "Evocation is not to be considered as diffusion in the usual sense of the term. The regions affected by organisine undergo profound modifications of their potencies. Metabolic gradients which hitherto have been latent are established and are conducive to the synthesis of organisine. There is, in other words, a sort of metabolic infection." And, later on (page 526 of his paper), Dalcq emphasizes the similarities between this "metabolic contagion" and the multiplication of genes and viruses.

These interesting ideas may be easily transplanted to a biochemical level. The first tangible transformation in the neural ectoderm at the time when induction takes place consists in the synthesis of ribonucleic acid, especially intense in the region of the interfaces which separate the neural ectoderm from the inductor. This ribonucleic acid is bound to granules whose number increases during development. Since there is hardly any doubt about this increase from fertilization to hatching, they must consequently undergo a multiplication. Now, in the chick embryo these granules can not be distinguished by chemical examination from a virus which causes



tumors in birds (Claude). In addition, the nucleoprotein character of the most widely differing viruses is generally admitted at present. There is thus nothing improbable in considering neural induction to be accompanied by a rapid increase in these particles in the nervous system under the influence of the organizer. This multiplication would then extend into other organs following precise gradients. A simple cytochemical examination of amphibian eggs suffices to show the course of this "contagion métabolique" pictured by Dalcq and Pasteels. In such a hypothesis, any agent able to initiate the multiplication of these nucleoprotein granules would be an evocator. It can easily be seen that the nucleotides necessary for the synthesis of these particles, the carcinogenic hydrocarbons and certain dyes which are selectively adsorbed, would rank among the best of the known evocators. Here again, one would obtain merely evocation if the multiplication of these granules were chaotic but if they increased in definite graded fashion then individuation would ensue. Naturally it is this latter alternative which would operate in normal development. We can easily see that slight changes in the chemical constitution of the granules, occurring little by little, would lead to the elaboration of new proteins and in this way new organs would appear. It is clear that the granules must undergo changes during development since we know that the granules isolated from various organs in the adult are chemically and serologically different. It would be very difficult to see how this would occur otherwise, or to suppose, for example, that the egg contained a mosaic of specific granules.

These ideas, some of which were developed earlier by Needham (1942), are very close to the hypotheses put forth by a number of workers to explain the origin of tumors (Potter, Woods and DuBuy, Graffi, Dittmar). Without entering into the details of these theories we see that the chief idea is the transformation of a normal nucleoprotein granule (microsome or mitochondria) into a virulent particle. For Woods and DuBuy, Graffi, and Dittmar, this would be a "malignant transformation" undergone by mitochondria. Potter envisages an enzymic complex resembling microsomes and thinks that an enzyme requiring the —SH groups for its functioning may be inactivated. This microsome with its enzymic activity thus reduced would then behave like a virus.

However, one can go still further and ask, as Haddow and Darlington do, if the nucleoprotein granules are not the basis of cyto-

plasmic inheritance, the reality of which has been demonstrated by Imai and Rhoades in plants and by Sonneborn in protozoa. Indeed, we know that the importance of cytoplasmic hereditary factors, long upheld by Goldschmidt and by Correns, tends to be confirmed more and more as a result of some interesting work of Spiegelman and Lindegren on the genetics of yeasts which have been adapted to ferment an abnormal sugar. Lindegren concluded from his experiments that the gene must be a double entity composed of a nuclear chromogene and a cytoplasmic cytogene. This hypothesis, very similar to those of Spiegelman and of Sonneborn, is valuable in explaining dominance and recessiveness, since the dominant gene would be the one having the greater affinity for the cytogene. But let us limit ourselves to the subject at hand, that is, the possible role of plasmagenes in morphogenesis. Rhoades, in particular, proposed the hypothesis that all of the cells of the organism have the same genetic constitution and that their differentiation is due to their cytoplasmic make up. The granules in the egg during development may behave like "plasmagenes," controlling the process of differentiation by insuring the synthesis of specific proteins. Furthermore Poulson came to this same conclusion after his beautiful research on the role of the X chromosome in the morphogenesis of *Drosophila*.

The idea that nucleic acids play a role in the phenomenon of cytoplasmic inheritance also comes from the careful experiments of Avery and his collaborators who have shown that rough type pneumococcus may be converted into smooth type by adding not the characteristic antigen of smooth type but rather its polymerized thymonucleic acid. Under the influence of the latter the antigen begins to be synthesized and the mutation is thus able to propagate itself. Similar examples of "directed mutations" under the influence of a specific thymonucleic acid have been observed in *Escherichia coli* by Boivin and his collaborators.

This second hypothesis complements quite well that which we have suggested here. There is no reason to suppose that "free" nucleic acids, independent of the granules, may not also undergo a hydrolysis into mononucleotides since we know that in yeast this fraction is more labile than the bound form. The visible synthesis of ribonucleic acid in the nervous system at the time of its formation may be an index of the multiplication of granules and, thus conceived, the two hypotheses form a harmonious whole.

It is the task of the future to determine how much truth lies in these suppositions which have the value of attempting to explain morphogenesis in physical and chemical terms amenable to experiment. This attempt at synthesis will not have been in vain if it stimulates new research. Problems to which little thought has been given also await solution, such as the role of particles comparable to the viruses during development, relations between the sterols and the synthesis of nucleoproteins, the multiplication of the granules, or the role of the changes which are undergone by the proteins during morphogenesis. These are the questions which are among the "perspectives in the field" into which we fear we have already entered too much.

### 3. Perspectives in the Field

#### (a) *Biochemical Significance of the "Morphogenetic Potentials"*

The main task of the future will be to find a common ground between experimental embryology and chemical embryology since it is only the fusion of these two disciplines which will permit a penetration into the mysteries of development. It is evident that every tentative explanation of morphogenesis is of interest to the biochemist. It is necessary, however, for the idea proposed to be based on exact information and to be sufficiently concrete to withstand the test of experimentation.

These conditions are fulfilled by the theory recently proposed by Dalcq and Pasteels which we will outline in general. The "morphogenetic bases" in the vertebrates are the vitelline gradient, with which we are familiar, and a field which has its center dorsally located and which coincides with the middle of the gray crescent in the frog egg. This field is not displaced by centrifugation nor by a rotation of the egg and thus Dalcq and Pasteels assume that it is anchored in the cortex of the egg, where it forms a film of proteins with associated lipids. Dalcq and Pasteels, in conformity with Schmitt's ideas, consider this cortical film to be a series of lamellar proteins arranged in parallel fashion and united by chains of lipids attached at right angles. According to these authors a chemical reaction occurs between the constituent C of the cortical field and the substance V of the vitelline gradient, the product CV formed reaching a different concentration at different regions of the egg. It is the amount of CV that will determine the fate of various regions

and especially their position during gastrulation. A supplementary factor, a "threshold" of concentration of CV, serves to explain the sharp change from one region to another, and the fate of the different cell groups is also basically influenced by the ratio C/V of the cortical and vitelline factors. From these various reactions the inducing substance "organisine" which is capable of autosynthesis arises. The sum total of these properties determines the "morphogenetic potential" of any particular region. This potential decreases, for example, from the notochord to the somites, to the pronephric primordium and to the lateral mesoderm. This concept has found support in experiments by Yamada, who, by grafting somite material near the lateral mesoderm, obtained an increase in morphogenetic potential of the latter which was transformed into pronephros.

If it is not yet possible, due to lack of sufficient facts, to discuss the Dalcq-Pasteels theory in detailed fashion, one can at least consider it in the light of known facts. The reality of a yolk gradient can not be doubted in the amphibian egg where numerous experiments have demonstrated the morphogenetic importance of the substance present in the vegetal region. Does this mean that the substance V is the same as yolk? Dalcq and Pasteels warn against such a conclusion and it is indeed difficult to visualize a chemical reaction, when one of the reactants is as complex and as large as a yolk platelet. In addition, we know that the yolk is inert from the point of view of respiratory metabolism and has no peptidase activity. However, when the oöcyte is homogenized and centrifuged the yolk layer liberates large amounts of inorganic phosphate by autolysis, as has been demonstrated independently by Harris and by Brachet. Thus it may be that the yolk platelets can lose a part of their phosphorus without breaking down. Indeed, Konopacki and Konopacka have reported that the yolk platelets lose their basophilia during development, probably by loss of their phosphate compounds. Perhaps these substances rich in phosphorus and sulfur contribute to the synthesis of nucleoproteins. We know, too, that the yolk in amphibians contains a little ribonucleic acid. These points should be studied systematically for it would be important to follow with appropriate methods the basophilia or acidophilia of the yolk in various regions of the normal egg and also after experimental procedures such as rotation and centrifugation. It would also be very valuable to compare the chemical composition of the large platelets situated at

the vegetal pole and the finer grains found at the animal pole, especially as to phosphorus content and the rate at which phosphate is liberated by autolysis. Recent observations by Brachet and his collaborators on the distribution of certain amino acids in the oöcyte have shown that the staining reactions given by these acids increases progressively from the animal to the vegetal pole. This fact makes it probable that the chemical composition of the yolk granules at the two poles is different. A chemical analysis of the small and large yolk granules is highly desirable and should be carried out soon in order to solve this problem definitely.

In regard to the cortical field of Dalcq and Pasteels, it is possible that it may be in part, at least, formed of ribonucleoproteins. Indeed, the frog oöcyte contains a strongly basophilic cortical layer which is not displaced by centrifugation. The subsequent changes in this layer have not been extensively studied, but it is clear that the membranes of the various blastomeres in the morula also contain ribonucleic acid. This acid has also been found in the cortical granules of the sea urchin egg by Runnström and Monné. Thus we see that the basic elements of the theory of Dalcq and Pasteels (vitelline gradient and cortical field) are recognizable cytologically and that there are even some indications as to their chemical nature.

The relations between the distribution of the CV substance and the sulfhydryl nucleoproteins are particularly striking, as pointed out by Dalcq and Pasteels. The amount of ribonucleic acid in any region, judging from a cytochemical examination, forms a true index of its morphogenetic potential. In Yamada's experiments, for example, the grafted somite material is richer in ribonucleoproteins than the lateral mesoderm, and the pronephros which forms between them and which has an intermediate morphogenetic potential is also intermediate, with respect to ribonucleoprotein content, to the somites and the lateral mesoderm. The importance of the dorsoventral gradient formed by these substances is particularly clear in this case. These observations serve as an outline for a vast program of study, since a large number of the classical experiments in embryology should be repeated and the changes in the ribonucleoproteins studied. We naturally think of grafts of organizers, heterotopic transplantations in various positions, translocations, etc. Such experiments will possibly lead to the determination of the nature of the thresholds and their value.

In addition there are other experiments that confirm the points in common between the hypothetical CV substance and the sulfhydryl ribonucleoproteins. The research of Brandes on localized irradiation of the organizer with U.V. led him to conclude that ultraviolet, depending on the dosage, either favored or inhibited induction. Relating these results to those of Uchimura on the redox potential and the glutathione content of irradiated muscle, Brandes formulated the hypothesis that weak stimulating doses increase the amount of —SH groups in the organizer. Stronger doses, on the contrary, are inhibitive because they cause a decrease in the —SH radicals. The results of Brandes, as well as those of Dürken, of Reith and of Schechtman, which show that ultraviolet radiation, especially at wave lengths where the nucleic acids absorb strongly, decreases the inductive powers of the organizer, argue in favor of a role for the nucleoproteins in induction. Pasteels has studied the effect of centrifugation on the development of the fertilized egg and obtained large deficiencies in the nervous system due to interference with induction which are easily explained if it is granted that the inducing substance is lighter than the yolk and is displaced toward the centripetal pole by the centrifuge, a phenomenon that occurs with the ribonucleoproteins—as determined by Pasteels and Brachet. We have seen above that the behavior of ribonucleic acid in eggs centrifuged at the blastula stage agrees well with the identification of ribonucleoproteins with the CV substance of Dalcq and Pasteels. All these findings confirm the idea that ribonucleoproteins, or the granules in which they are located, play an important part in the morphogenesis of the amphibian egg. Their identification with the CV substance thus becomes very probable.

Dalcq and Pasteels were especially concerned with an explanation of morphogenesis in vertebrates, but they also extended their theory to the invertebrates with certain modifications. In the sea urchin, the principal factor would be more the ratio between the animal and vegetal field  $An/Vg$  than the product of the reaction  $An \times Vg$ . Although it is too early to discuss this case, since it requires a special treatment, we may emphasize two facts. First, the necessity of maintaining a bipolar differentiation in order to obtain normal morphogenesis (Raven, Costello), a phenomenon which seems to be chiefly a separation of yolk from nucleoproteins. Thus the same factors appear as in the amphibian egg. However—and

this is the second point—we must recognize that at least in the sea urchin the yolk may be very different from that in the amphibian egg. While in the latter the yolk is inert, metabolically speaking, the centrifugable inclusions of the sea urchin egg contain the major part of the dehydrogenases and we know that this heavy material has a high respiration. This fact will need to be considered when an attempt is made to compare morphogenetic factors in echinoderms with those which we are beginning to recognize in the amphibians.

(b) *Nature of Fields*

We have not experienced much difficulty in demonstrating gradients in the vertebrate egg, but the situation becomes very complicated when we try to determine the chemical or physical nature

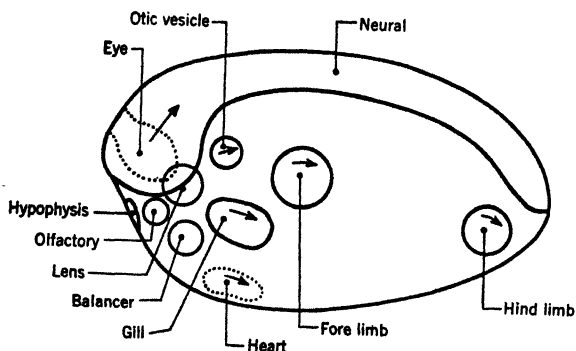


Fig. 124. Localization of various regional fields in the neurula of amphibians (Huxley and de Beer).

of fields. However, this concept has proved to be very fruitful in experimental embryology (Harrison, Holtfreter, Weiss, Dalcq and Pasteels *et al.*) although its basic significance still escapes us completely.

We have seen how Dalcq and Pasteels represent the primary cortical field in the egg, although it may be pointed out that F. E. Lehmann has recently cast doubt on the cortical localization of this field and tends to assign more importance to a subcortical plasm which is concentrated in the dorsal half after fertilization (Fig. 87). The distribution of this plasm is modified when the egg is rotated 180° and its chemical analysis should not be delayed.

A microscopic examination reveals no peculiarities in the region of the different fields recognized by Harrison and his school (lens field, otic vesicle field, limb bud field, etc.) (Fig. 124). The use of cytochemical techniques has given no better results. In the case of the sulfhydryl ribonucleoproteins, a local synthesis is found at the time of differentiation of organs but there is no evidence of this before organogenesis. The question should be studied very carefully.

We are indebted to Harrison for an interesting hypothesis on the nature of fields, that is that these regions may possibly be distinguished by the structure of the proteins which make them up. The chemical changes occurring in the embryo may be accompanied by rearrangements in the structure of proteins. There may also be forces of crystallization which insure gastrulation and neurulation by altering the form of the molecules by producing new tensions and finally by stimulating deformation of the cell. Harrison terminates his article by suggesting a study of the differentiating embryo by x-ray diffraction methods; perhaps in this way one could gather some information on the structure of embryonic proteins.

Harrison has approached this problem experimentally by collaborating with Astbury, a specialist in the x-ray analysis of protein structure, and Rudall. A method permitting the x-ray examination of polarized primordia such as the ear and limb was tested, but the results were unfortunately negative in the sense that no orientation of molecules could be recognized in the photographs.

This failure does not suffice to eliminate Harrison's hypothesis, for the demonstration of molecular orientation in such a complex material is subject to great difficulties. There is nothing to rule out the idea that most of the molecules are oriented at random, but that certain of the most important ones from the point of view of morphogenesis are disposed in parallel fashion. The work of Harrison, Astbury and Rudall has not been in vain because they have made possible the application of a method which will certainly render a great service in other fields or with other material. In any case it is not impossible that fields correspond to regions in which the protein molecules have a special structure which our present gross methods of investigation can not discern. Perhaps continual refinements in the electron microscope may answer this problem in the future.



(c) *Biochemical Basis of Organogenesis and of Histological Differentiation*

These questions, like the preceding, have not received the attention they deserve up to the present. This is not surprising because primary morphogenesis has raised enough urgent problems by itself to claim the attention of numerous investigators. It is clear that the formation of organs and their cellular differentiation must be accompanied by some basic physical and chemical changes which sooner or later will have to be considered in chemical embryology. One might predict that the formation of specific proteins plays an important role in the "chemodifferentiation" (Huxley) of organs and the molecular rearrangements of proteins are probably among the essential agents of organogenesis. In any case, the synthesis of new proteins during development appears clearly in the immunological investigations of Braus, of Mollison, and of Avrech and Heronimus, who have shown that the adult contains antigens which are lacking in the egg. On the other hand it is important to recognize that the frog egg already contains some of the antigens present in the serum of the adult (R. S. Cooper) and the same situation was found in the chicken by Schectman. Without doubt immunological methods will render great service to chemical embryology now that the techniques have been adapted to work on the amphibian egg by R. S. Cooper. It would appear that it is those organs with a particularly characteristic morphology, physiology and biochemistry that can be attacked with most success in the study of chemodifferentiation. The differentiation of the somites into striated muscle would probably be most susceptible to analysis. Several trials have already been made in this direction and have furnished some interesting, if incomplete, results. Thus J. Needham, D. M. Needham, Yudkin and Baldwin, then Baldwin and D. M. Needham have studied phosphagen during development in the squid and the chick. There is a clear relation between the value of the ratio:

$$\frac{\text{P of phosphagen}}{\text{P of phosphagen} + \text{P inorganic}}$$

and the degree of differentiation of muscle tissue. In the chick the amount of phosphagen increases sharply on the 14th day of development when the head and trunk show movements and attains a maximum when the first contractions of the limbs and tail begin. In

the frog, Zielinski (1935) showed that the egg contains phosphocreatine at fertilization, certainly well before the appearance of muscles. This compound is rapidly synthesized, as in the chick, at the time of the first muscular contractions. Synthesis becomes more intense as the movements increase, so phosphocreatine must play the same role in the muscle of the embryo as it does in that of the adult. Similar investigations might be advantageously extended to other chemical compounds which take part in muscular contraction such as adenosine triphosphate, adenylypyrophosphatase, myosin, and the ions  $K^+$ ,  $Mg^{++}$ . This research would be especially striking if it were to be carried out on isolated embryonic muscle restricted to the somites instead of including the whole embryo. Such problems are technically accessible at the present time and an interesting beginning has very recently been made by Nicholas and his co-workers.

The appearance of cholinesterase in the nervous system of the embryo has been the object of interesting studies, Nachmansohn showed that this enzyme is synthesized during the development of the chick in direct relation to the appearance of the synapses and nerve endings. In the amphibian (Youngstrom, Artemow, Sawyer), cholinesterase can be detected in an early stage even before the embryo shows movements. However, there is a rapid synthesis, later, just as in the grasshopper (Tahmisian) where the appearance of the enzyme coincides with the differentiation of nerve cells. The most important experiments along these lines are those of Boell and Shen. Determining cholinesterase by means of the diver, these authors showed that as early as the closure of the neural folds the latter have a higher content of the enzyme than the epidermis. Subsequently the difference in this respect between these two tissues becomes very great. When a secondary nervous system is induced by an organizer graft it also becomes as rich in cholinesterase as the nervous system of the host and can be distinguished in this way from the epidermis which has not been stimulated by an inductor. Determinations of cholinesterase in ectoderm subjected to an evocator might eventually permit a more rapid, quantitative, and certain distinction between true neural evocations and neuroid reactions than a morphological study.

Let us now consider the interesting contribution by F. Moog and S. Flexner to the problem of chemodifferentiation. F. Moog carried out an important study on the distribution of acid and alkaline

phosphatases in the chick embryo by using both the cytochemical method and quantitative techniques simultaneously. Without entering into the details of the results obtained, it may be noted that Moog came to the conclusion that the amount of phosphatase attains a maximum in every organ at the time of its differentiation. This result is verified clearly in amphibian eggs (Brachet, Moyson), especially in the case of alkaline phosphatase which is found for the most part in the nuclei. The presence of a coupling between alkaline phosphatase and the synthesis of proteins also appears from the cytochemical investigations of Bradley and of Jeener. The latter observed a considerable increase in the intensity of the alkaline phosphatase reaction in the vagina of ovariectomized animals when they are injected with hormones. It is interesting to recall in this respect that there is a remarkable parallel between the amount of alkaline phosphatase in the nuclei and the rate of turnover of phosphorus in thymonucleic acid. In what manner is the renewal of phosphorus in thymonucleic acid coupled with the synthesis of proteins? We still do not know, but one can not resist relating these observations with those of Spiegelman and Kamen showing that the synthesis of proteins in yeast is accompanied by a decrease in phosphorus in the nucleoprotein fraction. It may be, as Spiegelman and Kamen think, that the nucleic acids form the specific energy donors during protein synthesis. It is clear that a thorough study of phosphate metabolism during the period of differentiation in the embryo is very necessary in the near future.

With regard to Flexner's work it shows that the amount of respiratory enzymes (cytochrome oxidase, succinic dehydrogenase) of the kidney, and of the cerebral cortex increases greatly when these organs differentiate and become functional. In view of the close coupling between these two enzymes and the microsomes it would be well to see if an increase in the number of these granules occurred at this time or whether there was an increase in the amount of the two respiratory enzymes in the particles. Here again we emphasize the ties between the respiratory enzymes in the microsomes and the synthesis of specific proteins.

Let us recall a fact previously reported which may throw some light on the phenomena of organogenesis and differentiation. Basophilia, in other words the amount of ribonucleic acid, greatly increases in each organ at the time of its formation and decreases subsequently

when cytological differentiation is achieved. There is some very clear evidence of this in the embryos of the vertebrates (Brachet, Caspersson and Thorell). The ribonucleic acid of the notochord increases rapidly during neurulation, but basophilia decreases when the cells become vacuolated. Furthermore, the amount of this acid in the somites is greatly increased at the time when the muscles form and becomes less when striations appear. Similarly the nervous system is very basophilic in the neurula stage and gradually loses nucleic acids in subsequent development. This does not occur in the digestive tract or glands where one does not observe a secondary diminution of basophilia, a normal situation, since these organs secrete proteins in great quantities in the adult and are rich in ribonucleoproteins. It is furthermore very striking that the digestive tube, the liver, and pancreas differentiate very late and their primordia are also the last to synthesize nucleic acid in abundance. Thus it appears that organogenesis goes hand in hand with a synthesis of ribonucleoproteins, probably linked with the formation of specific proteins. When cytological differentiation attains its maximum, ribonucleic acid tends to disappear, since the characteristic proteins of each organ have been formed at this time so that synthesis slows down.

The pursuit of these studies certainly will contribute to the increase in our knowledge of the mechanism of protein synthesis. One may well ask, for example, whether the nucleoprotein granules in the somites become modified at the time when they begin to form myosin. This is one of the kinds of questions still to be answered. Perhaps such research would clarify some of the problems which embryology has attempted to resolve for a long time. For instance, does the fertilized egg already contain all the characteristic proteins of the different organs, or are these synthesized little by little in the various regions? This is one of the aspects of the eternal discussion between the partisans of preformation and epigenesis which must be considered. The actual findings tend to show, it seems to us, that the synthesis of specific proteins begins only when the basophilia of the primordia increases sharply. We may also hope that the progress in this field will clarify other important points such as the "determination" of embryonic regions. The program before us is vast, not to say inexhaustible.

(d) *Genes, Morphogenesis and Biochemistry*

At the present time there is no question that genes play a role in the regulation of embryonic development. This is a point which is important to recognize although Harrison rightly warns against purely genetical theories of ontogenesis. Any concept that neglects cellular rearrangements and the organization of the egg cytoplasm is hopelessly one-sided and incomplete. As Just very aptly pointed out (cited by Harrison, 1937) the embryologist is far more interested in the mode of formation of the eye than in the way in which they acquire color. Nevertheless, it will be remembered that Ephrussi, Khouvine, and Chevais undertook a careful study of the morphogenetic role of genes which control the formation of the eye in *Drosophila* by an analysis of the *bar* gene. This work has been followed successfully by Chevais who has assembled all the preliminary factors for an attack on the problem from a chemical angle. An important result has already been established in this regard, for a recent note by Y. Khouvine, S. Chevais and J. Grégoire announces that the substance which increases the number of eye facets in *bar* eye mutant is an imidazol derivative and may be I-methyl-hydantoin.

It is clear that the action of genes in development and their manner of action in the metabolism of the embryo will be important fields of research in the future. It will be recalled that an experimental analysis has already been begun by Poulson in *Drosophila*, demonstrating that the X chromosome controls the migration of the nuclei during segmentation and the gastrular movements. The absence of the X chromosome has no effect on the oxygen consumption of the egg until the time when the abnormalities of development arise (Boell and Poulson). From this it is concluded that the primary action of the genes is not accompanied by changes in the rate of oxidation.

The manner in which genes control embryonic development would be clarified if we knew more about their nature and mode of operation in the more accessible cases. In this respect, the beautiful studies of Scott-Moncrieff on the pigments which produce the colors in flowers and especially those on the pigmentation of the eye in *Ephestia* and *Drosophila* have already contributed to progress in this field. Without discussing these investigations, which are very well treated in a recent book by Guyenot, let us mention some of the research by Caspari, Beadle and Ephrussi, Ephrussi and Beadle, and

Tatum and Beadle which have led to the isolation of an intermediate between the vermilion gene ( $V^+$ ) and the eye pigment. This substance formed under the influence of the gene diffuses into the internal fluids and circulates like a hormone entering into a chain of reactions which results in the formation of the vermilion eye pigment. Work by Butenandt, Weidel, Weichert and von Derjugin has shown that it is a derivative of tryptophan, kynurenin. In regard to the nature of genes it will be recalled that the experiments of Kuhn and Moewus render very probable the identity of the masculinizing gene in *Chlamydomonas* with an enzyme which hydrolyzes picrocrocin.

It is with great enthusiasm that one views the recent developments in biochemical genetics in the United States. Its remarkable success is due in great part to the choice of microorganisms as material. Even a summary analysis of the intensive investigations of Beadle and his school on *Neurospora*, of Sonneborn on *Paramecium*, of Spiegelman and Lindegren on yeast, and of Delbrück, Cohen *et al.* on bacteriophages would take us too long. Without doubt this fundamental work will one day constitute the basis of research in chemical genetics of the embryo and it may be predicted that it will have deep repercussions on the thinking of embryologists.

A first step which indicates the path to be followed has been taken by Gordon and Sing in a study of the mode of action of a gene influencing the morphogenesis of an organ, the formation of the antennae in *Drosophila*. Gordon and Sing subjected homozygotes for the gene *antenna-less* to various nutritive media. They found that a diet rich in vitamin  $B_2$  permits the growth of the antennae and thus it appears by analogy with the results of Beadle in *Neurospora* that the mutant gene *antenna-less* is not able to bring about the synthesis of a specific chemical (similar or identical to  $B_2$ ) necessary for the formation of the antenna. Schultz's work on the culturing of *Drosophila* in a synthetic medium will probably make further research of this kind feasible.

But however promising these beginnings, one can not as yet formulate a hypothesis on the manner of the action of genes in primary morphogenesis. The work of Baltzer and his school indicates the direction to follow. Baltzer has studied chiefly the effects of simple and merogonic hybridization (removal of the female nucleus) on the development of urodeles. The results vary according to the combinations used but it is clear that morphogenesis stops

earlier in merogonic than in simple hybrids. Thus one can expect only poor development when the egg has only a single haploid nucleus of another species. The role assumed by the nuclear factors is also shown by the experiments in which the fertilizing spermatozoön is treated with radium, x-rays, ultraviolet or trypanflavin (G. Hertwig, Dalcq). Dalcq and Simon have especially emphasized the importance of the nucleus for gastrulation. Indeed in many hybrids of anuran species development stops when the dorsal lip of the blastopore forms (see P. Hertwig). The same observation was made by J. Moore to whom we owe a careful and detailed study of the various hybrid combinations of the anurans of the United States. Again he often found a blocking of development at the gastrula stage. When this critical stage is passed development generally proceeds to metamorphosis. It is particularly interesting to note that, according to Moore, the rate of development follows the maternal rate up to the gastrula stage in all the combinations studied. In the hybrids which pass this stage the paternal influence is exerted on the rate of development only after gastrulation. Moore concludes from this that development up to gastrulation is governed by cytoplasmic factors while subsequent differentiation is dependent upon specific interaction between the nucleus and cytoplasm.

Finally, it will be recalled that the older studies of A. Brachet and of M. Herlant tell us that the viability of haploid embryos is less than that of their diploid partners, while polyspermic larvae die even earlier. In this last case, the haploid and diploid nuclei form a mosaic and it is this nuclear heterogeneity to which we must attribute the cause of death. There are in addition certain merogonic hybrids in which Fankhauser has found that the number of chromosomes in the blastomeres varies from 3 to 44. It is hardly necessary to add that development is rapidly arrested under these circumstances.

An experimental analysis of the lethal factors in merogonic hybrids with a uniform haploid constitution has been undertaken in Baltzer's laboratory. We will pay particular attention to results of Hadorn who removed fragments of ectoderm from the lethal hybrids at the gastrula stage and transplanted them to a normal gastrula. While the graft would have died in the neurula stage without this operation, it is able to develop perfectly when transplanted, so one must conclude that the contact with a healthy diploid host causes the lethality due to hybrid merogony to disappear. Comparable findings were

obtained by Baltzer, Schönmann, Luthi and Boehringer for the strongly lethal combination *Triton palmatus* ♀ × *Salamandra maculosa* ♂ and the corresponding merogon in which the nucleus is removed from the egg of *Triton palmatus*. The diploid hybrid undergoes nuclear disarrangements at the blastula stage, characterized by the appearance of numerous abnormal mitotic figures. If a fragment of the ectoderm of this hybrid is transplanted to a normal diploid gastrula of *Triton palmatus* it differentiates according to its new position (*Ortsgemäss*) in the host and gives rise to various types of organs. The authors assume that the healthy host produces a substance necessary for morphogenesis which is not formed in the hybrids, this substance arising from the interaction of the nucleus and cytoplasm of the same species.

Brachet (1945) obtained similar results with the hybrid *Rana fusca* ♀ × *Rana esculenta* ♂ in which development is always arrested at the beginning of gastrulation. He explanted a fragment of the organizer instead of a piece of ectoderm, and grafted it into the blastocoel of the gastrula of *Triton*. In most cases the organizer graft went on developing and differentiated into absolutely normal notochord. At the same time it induced a typical secondary embryo provided with a nervous system showing regional differences and flanked by somites. When the implanted blastoporal lip of the hybrid is blocked its power of induction appears to be reduced. Thus it is seen that the lethal factors disappear following the transplantation and the blastoporal lip acquires its full inductive power and capacity for self-differentiation into notochord. These experiments prove in addition that the substance lacking in the hybrid merogon is not specific since development of a fragment of a frog hybrid regains its activity upon transplantation into *Triton*.

In the comparable hybrid combination *Rana pipiens* ♀ × *Rana sylvatica* ♂, Moore found also that the organizer from the blocked gastrulae resumes its inducing activities—although in a somewhat weakened way—when it is grafted into a normally developing embryo.

It is also known that the favorable effect of transplantation of lethal tissue into a healthy host is also shown in more advanced stages. Thus Gayer, then Gayer and Hamburger, extirpated the optic primordium of a strongly lethal mutant in the chick (creeper fowl), in which the abnormalities, chiefly of the eye and limb, are



due to a lethal gene, as Landauer has established in his careful studies. This eye, which should present gross abnormalities, differentiates normally when transplanted into the optic region of an embryo lacking the lethal gene. The same results are obtained with limb regions of the creeper fowl when they are transplanted into a genetically normal embryo.

Let us mention finally a very good study by Porter on the interaction of nucleus and cytoplasm in androgenetic eggs (deprived of the egg nucleus by puncture during maturation, and then fertilized) of *Rana pipiens*. Porter used northern and southern races and subjected them to different temperatures. The development of the northern race shows great acceleration when the individuals are raised at the temperature of the southern race, and, conversely, if the latter is cooled development is greatly retarded. If the androgenetic experiments are performed similarly and the combination of "southern cytoplasm and northern nucleus" is observed it is found that the embryos at high temperature show differences in acceleration, the anterior half becoming hypertrophied. In the case of the reverse combination of "northern cytoplasm  $\times$  southern nucleus" mitoses in the anterior region are retarded and this part is proportionately reduced.

Certain authors have attributed the precocious arrest of development of diploid and merogonic hybrids to a tardy utilization of the yolk. A similar situation would seem to obtain in parthenogenetic embryos (G. Hertwig, Porter). These facts led Brachet (1945) to investigate the possibility that the synthesis of ribonucleic acid is inhibited in parthenogenetic, polyspermic, and hybrid embryos. We have seen, in effect, that the ribonucleoproteins are synthesized at the expense of the yolk and we know in addition that ribonucleic acid very probably plays a role in morphogenesis. One may then ask whether the lethality of larvae with abnormal nuclear constitution may not be due to an inhibition of the synthesis of ribonucleoproteins. This hypothesis is the more plausible since we know of the direct relationships between the two types of nucleic acids. It will be recalled especially that the synthesis of ribonucleoproteins in the oöcyte of *Drosophila* seems to be conditioned by the chromosomal formula of the nuclei according to Caspersson and Schultz (although recent pentose analyses by Callan throw some doubt on this conclusion).

The principal results of this study are a marked retardation in the

synthesis of ribonucleic acid in the parthenogenetic tadpoles every time they show hypoplasia. When the haploid region of a polyspermic embryo is less differentiated than the remainder it also has a much lower ribonucleic acid content. When we examine any of the hybrid embryos studied which have been blocked at gastrulation, we see that the synthesis of ribonucleic acid is arrested and that this synthesis recovers, as does development, after transplantation into a healthy host. We thus see confirmed once more the direct coupling between morphogenesis and the formation of ribonucleoproteins. Naturally, one can not yet attribute the blocking of development to disturbances in nucleoprotein metabolism but there is nothing which would obviate such an explanation. Here again a field of tremendous experimental possibilities opens up and the whole biochemistry of lethal hybrids remains to be explored. It would be especially interesting to know if the frequent blocking of embryos in the young gastrula stage is due to the fact that carbohydrate metabolism, which is linked with that of the nucleic acids, can not begin. All that we know in this respect is that the oxygen consumption of blocked gastrulae with nuclear abnormalities is lower than normal (personal observations of Brachet cited by Dalcq and Simon).

Recently Barth (1946) has published results of a study of the metabolism of a hybrid in which development is blocked at gastrulation. This is a combination of *Rana pipiens* ♀ × *Rana sylvatica* ♂, the development of which had been studied by J. Moore. Barth's research, whose importance can not be underestimated, deals with the oxygen consumption, the respiratory quotient and the anaerobic lactic acid production, with the following results.

The oxygen consumption at first increases in the same way as the controls (eggs of *Rana pipiens* fertilized by the sperm of the same species) but this increase stops at the time when development is blocked, while it continues, naturally, in the controls. This result is in short identical with that of Boell and Poulson on the eggs of *Drosophila* lacking the X chromosome and it supports the ideas of Tyler that energy is necessary for morphogenesis.

The measurements of the respiratory quotient carried out by Barth show that the metabolism of the hybrids does not differ qualitatively from that of normal embryos. The R.Q.'s of the two types of embryos remain between 0.85 and 0.90 during all of development. It should be noted in passing that the amount of CO<sub>2</sub> chemically

combined in the form of bicarbonate decreases at the end of gastrulation in the controls but continues to increase in the hybrids, which results from the fact that the archenteron fluid, rich in bicarbonate, as shown by Gregg, is expelled by control embryos but not by hybrids.

As to anaerobic lactic acid production, it is less in the hybrids than in the controls at the beginning of gastrulation when the block to gastrulation occurs and does not increase with further development so that the curves for oxygen consumption and anaerobic glycolysis are parallel.

Barth interprets his results in the following manner. The respiratory block may be due to the fact that a precursor of lactic acid does not form in sufficient amounts, but it can not be caused by a deficiency in the hydrogen transport system. Everything points to the foreign chromosomes of *Rana sylvatica* as the producers of a substance inhibiting oxygen consumption. Probably a substance necessary for the oxidation of all of the substrates present in the egg becomes a limiting factor in the early gastrula so that further development is rendered impossible.

One may also ask whether the metabolism of lethal hybrids is not conditioned by their inability to synthesize or permit the multiplication of microsomes. Indeed, it will be remembered that these granules contain the ribonucleoproteins and respiratory enzymes (succinic dehydrogenase and cytochrome oxidase) together. During normal development the amount of ribonucleic acid increases (Brachet) and the same is true for these two respiratory enzymes (Boell). We have seen earlier that there is a remarkable parallel in the amphibian egg between oxygen consumption and the ribonucleic acid content of different regions. We also know that Boell considers the synthesis of cytochrome oxidase and succinic dehydrogenase to be an index of the transformation of yolk into active cytoplasm and we have seen that the utilization of yolk is retarded in embryos where the chromosomal balance is abnormal. If we put all these facts together, the idea emerges that the multiplication of the microsomes must be under the control of the chromosomes. Such a hypothesis, which certainly needs experimental verification, falls in perfectly with the observations of Caspersson and Schultz on the amount of ribonucleic acid in the oöcytes of *Drosophila* having different genetic constitution.

Our task is done. We have tried to give an accurate idea of the present tendencies in chemical embryology by assembling in this somewhat condensed treatise the multitude of facts which confront us. We have attempted to point out the essential results, which are often contradictory and have not dismissed without some discussion the gaps in our knowledge or the controversial points. The critical attitude which we have adopted, in places, is justified by the fact that the young science considered in this book can be successfully developed only if it rests on a firm foundation. In order to stimulate its progress we have, from time to time, suggested some experiments designed to clarify doubtful points. We will have attained our end if the interest of the reader is sufficiently stimulated so that he feels the urge to contribute to this common endeavor.

We should not conceal the fact that such a treatise lacks the integration and harmony appertaining to a more mature discipline. Here, all this new material may be integrated rather arbitrarily, but we hope to have shown that it is full of promise. The progress attained by chemical embryology in the last ten years, thanks to the increasing refinements of techniques, is really surprising and presents today a wealth of information. The time draws near when chemical embryology will reveal to us the basic significance of the gradients, the fields and the potencies which the experimental embryologist has given us. But already it has contributed much to the solution of the puzzles of morphogenesis and on this level it is fully integrated into that vast domain of causal embryology as conceived by Albert Brachet.



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